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(54) Title: PEGYLATION OF POLYPEPTIDES

(57) Abstract

Compounds are disclosed having the general formula R_1-X-R_2, wherein R_1 and R_2 are biologically active groups, at least one of which is polypeptidic. X is a non-peptidic polymeric group. R_1 and R_2 may be the same or different. Preferred R_1 and R_2 groups are interleukin-1 receptor antagonist, 30kDa TNF inhibitor, interleukin-2 receptors and CR1 and muteins thereof. Also included are site selectively modified interleukin-1 receptor antagonist and 30kDa TNF inhibitor.

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PEGYLATION OF POLYPEPTIDES

FIELD OF THE INVENTION

This invention relates to polypeptides that have been covalently bonded to long chain polymers such as methoxy polyethylene glycol. This invention also describes methods and reagents for the reaction of activated polymer molecules with various biologically-important polypeptides.

BACKGROUND OF THE INVENTION

Many proteins that have been identified and isolated from human and animal sources have been found to show promising medicinal or therapeutic potential. Great strides have been made in the methods for identifying and characterizing such proteins, in addition to methods for producing such proteins in relatively pure forms and relatively large quantities. As the development process advances in relation to the utilization of such potentially valuable materials, many obstacles have arisen in formulating these compounds for use in clinical models.

For example, many such proteins have been found to have an extremely short half life in the blood serum. For the most part, proteins are cleared from the serum through the kidneys. The systematic introduction of relatively large quantities of proteins, particularly those foreign to the human system, can give rise to immunogenic reactions that, among other problems, may lead to rapid removal of the protein from the body through formation of immune complexes. For other proteins, solubility and aggregation problems have also hindered the optimal formulation of the protein.

One of the most promising techniques for addressing these problems has been covalently bonding one or more inert polymer chains to the polypeptide of interest. The most commonly used polymer is polyethylene glycol (PEG), or monomethoxyl polyethylene

glycol (mPEG). See, for example, Davis et al., Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, pp. 441-451 (1980). PEG is ideal for these purposes due to its proven non-toxic properties. Other researchers have utilized polyoxyethylated glycerol (POG) for similar purposes. Knauf et al., J. of Biolog. Chem. vol. 263, pg. 15064 (1988).

Numerous results have been described whereby the covalent modification of proteins with polyethylene glycols ("pegylation") have resulted in the addition of desirable characteristics to the protein. For example, the pegylation of IL-2 has been shown to decrease the clearance of IL-2 while not significantly affecting the activity of the cytokine. The decreased clearance leads to an increased efficiency over the non-pegylated material. Katre et al., Proc. Natl. Acad. Sci. U.S.A. vol. 84, pg. 1487 (1987).

Increasing the half-life of Superoxide Dismutase (SOD) in blood serum has been a critical barrier for the use of SOD for the treatment of various symptoms. A number of studies have shown that the pegylation of SOD will give rise to a decreased clearance rate. See, for example, Conforti et al., Pharm. Research Commun. vol. 19, pg. 287 (1987).

Aggregation of Immunoglobulin G (IgG) has been postulated as a factor that leads to serious side effects to patients that are intravenously administered IgG. It has been shown that the pegylation of IgG reduces the aggregation of the proteins to prevent this problem. Suzuki et al., Biochem. Biophys. Acta vol. 788, pg. 248 (1984).

The ability of pegylation techniques to affect protein immunogenicity has also been shown. Abuchouski and coworkers have studied the immunogenicity and circulating life of pegylated Bovine Liver Catalase. Abuchowski et al., J. Biol. Chem. vol. 252, pg. 3582

(1977).

The addition of PEG groups to these various proteins decreases clearance due to the increase in molecular size of the pegylated protein. Up to a certain size, the rate of glomerular filtration of proteins is inversely proportional to the size of the protein. The ability of pegylation to decrease clearance, therefore, is generally not a function of how many PEG groups are attached to the protein, but the overall molecular weight of the altered protein. This has been borne out by clearance studies that varied both the size of the PEG side chains and the number of PEG units bonded to IL-2. Katre, supra.

The various studies of pegylated proteins in relation to clearance, immunogenicity, aggregation and physical properties all suggest that the PEG forms a flexible, hydrophilic shell around the protein. The PEG chains become highly hydrated and give the pegylated proteins a higher apparent molecular weight than would be predicted, and act to shield charges on the protein.

Because of the many promising results that have been seen in this field, a catalogue of procedures for the attachment of PEG units to polypeptides has been developed. The key element in these procedures is the "activation" of the terminal-OH group of the polyethylene glycol. Such activation is necessary in order to create a bond between the PEG group and the polypeptide. The vast majority of coupling procedures activate the PEG moiety in order to react with free primary amino groups of the polypeptides. Most of these free amines are found in the lysine amino acid residues.

In general practice, multiple PEG moieties are attached to the proteins. For example, in United States Patent No. 4,179,337 of Davis et al., it was found that to suppress immunogenicity it is desireable

to use between 15 and 50 moles of polymer per mole of polypeptide.

Because multiple PEG chains are generally bonded to each polypeptide, and because there are typically a large number of lysine residues in each protein, there has been little effort to pegylate proteins to yield homogenous reaction products. See, Goodson et al. Biotechnology, vol. 8, pg. 343 (1990); U.S. Patent No. 4,904,584 of Shaw. This lack of reaction specifity gives rise to a number of complications. Among these, are that pegylation often results in a significant loss of activity of the protein. Presumably, attachment to a critical lysine residue could alter the active site of the protein rendering it inactive.

It has been shown in at least one system, that pegylation can lead to sterically hindered active sites. In other words, relatively small substrates may approach the protein, while the activity of proteins that react with larger substrates can be dramatically effected by random pegylation. Davis et al. supra. The site selective pegylation of such proteins could lead to modified materials that gain the desireable attributes of pegylation without the loss of activity. In addition, if the pegylated protein is intended for therapeutic use, the multiple species mixture that results from the use of non-specific pegylation leads to difficulties in the preparation of a product with reproducible and characterizable properties. This makes it extremely difficult to evaluate therapeutics and to establish efficacy and dosing information.

In certain cases, it has been found that the administration of multimeric complexes that contain more than one biologically active polypeptide or drug can lead to synergistic benefits. For example, a complex containing two identical binding polypeptides may have substantially increased affinity to the ligand

or active site that it binds relative to the monomeric polypeptide. For this reason, multimeric complexes of proteins can be desirable in order to increase affinity of the protein to its ligand in addition to increasing the molecular weight of the complex.

Proteins frequently achieve their biological effects through interaction with other proteins. Where a simple complex of two proteins is sufficient to achieve the biological effect it has proved possible to mimic the physiological effects of endogenous proteins by administering exogenous proteins. However, where the biological effect requires the assembly of a complex containing more than two proteins it is more difficult to mimic the function of the endogenous proteins with recombinantly produced exogenous equivalents because the higher order complexes are frequently unstable. In such cases it may be advantageous to use crosslinked species containing two of the components of the complex to simulate the biologically-active complex.

Subsequent to the invention described herein, at least three research groups have described the production of crosslinked proteins, where the extracellular portions of one of the TNF receptors is attached to the heavy chain of human or mouse IgG, which are then crosslinked through disulfide bonds. Peppel et al., J. Exp. Med. vol. 174, pg. 1483 (1991); Ashkenazi et al., Proc. Natl. Acad. Sci. USA vol. 88, pg. 10535 (1991); and Loetscher et al., J. Biol. Chem. vol. 266, pg. 18324 (1991). In each case, the proteins were expressed in animal cell expression systems, and were found to be substantially more effective at inhibiting TNF than the monomeric soluble receptor alone. Similar procedures have also been used for producing similar crosslinked proteins of the CD4 protein, (Byrn et al., Nature (London) vol. 344, pg. 667 (1990)) the CR1 protein, (Kalli et al., J. Exp.

Med. vol. 174, pg. 1451 (1991); Hebell et al., WO 91/16437 (1991)) and the CR2 protein. (Hebell et al., Science, vol. 254, pg. 102 (1991)).

These crosslinked proteins -- constructed of two polypeptide units and a portion of the IgG antibody -- have been shown to have promise as therapeutic agents. The crosslinked proteins have an increased molecular weight, which acts to decrease the rate of clearance of the complex from the body, in addition to the apparent enhancement of the affinity of the proteins to their ligand. However, the proteins crosslinked in this manner have so far only been prepared by expression in animal cell expression systems by the expression of fused genes. This has been required in order to have the IgG portion of the protein properly folded after expression. In addition, the fixed heavy chain portion of the IgG antibody that serves as the spacer or linker between the polypeptide units does not allow for the ability to vary the length, size or geometry of the spacer. Given the apparent synergistic effect achieved by the dimeric proteins, it is likely that by varying the spatial orientation of the polypeptides the synergistic benefit may be optimized. And finally, the crosslinked proteins may be antigenic and/or have decreased solubility. The heavy chain of antibodies is not biologically inert.

Other dimeric or "bivalent" complexes have been described. One such group of dimeric compounds has been labeled hirulogs. These compounds are comprised of very short polypeptide units that are linked by a short poly-glycine spacer or linker. One of the polypeptide units is a thrombin inhibitor -- a 5 amino acid sequence taken from the 65 amino acid protein Hirudin -- and the other is an anion-binding exocite (ABE) recognition inhibitor. See, Maragonore et al., Biochemistry, vol. 29, pg. 7085 (1990); Bourdon et al.,

FEBS vol. 294, pg. 163 (1991).

C-reactive protein (CRP) is an acute phase serum protein composed of five identical 23kDa subunits. CRP can induce reactions of precipitation and agglutination and can also react with Clg to activate the classical complement pathway. Cross linked oligomers of CRP have been formed using bis (sulphosuccinimidyl) suberate or 3,3'-dithio (sulphosuccinimidylpropionate) as cross-linking agents. Jiang et al., Immunology vol. 74, pg. 725 (1991).

The formation of dimeric or bivalent ligands for targeting opoid receptors has also been investigated. Non-peptidic β-naltrexamine or oxymorphamine pharmacophores have been connected by short ethylene oxide or glycine spacers. Erez et al., J. Med. Chem. vol. 25, pg. 847 (1982); Portoghese et al., J. Med. Chem. vol. 29, pg. 1855 (1986). Tetrapeptide enkephalins linked by short methylene bridges have also been designed to target opoid receptors, and have been shown to have a greater selectivity and affinity for the delta receptor than the original delta ligand. Shimohigashi et al., Nature vol. 197, pg. 333 (1982).

The cell surface glycoprotein CD4 has also been produced in multimeric forms through a sugar-based cross-linking strategy. The cross-linking agent utilized was bismaleimidohexane (BMH). Chen et al., J. Biol. Chem. vol 266, pg. 18237 (1991).

Lymphocyte function-associated antigen-3 (LFA-3) is a widely distributed cell surface glycoprotein that is a ligand for the T lymphocyte CD2. LFA-3 with its associated lipids forms protein micelles of eight monomers which increased their ability to interact with cells with CD2 on their surface. Dustin et al., J. Exp. Med., vol. 169, pg. 503 (1989).

In a somewhat related technology, one group has studied the inhibitory effect of a synthetic

polypeptide that is comprised of a repeating pentapeptidyl unit. The polymer was synthesized by the polymerization procedure with diphenyl phosphoryl azide to a size of about 10,000 daltons. The polymerized pentapeptide is one of the essential structures in several biological responses. Morata et al., Inst. J. Biol. Macromol. vol. 11, pg. 97 (1989).

A further obstacle in developing effective exogenous proteins to augment or compete with endogenous substances is that exogenous proteins must be administered systematically rather than being localized in the appropriate place. This can lead to lower efficacy and to increased side effects. Several groups have reported targeting bioactive proteins to the appropriate sites by linking them to other proteins that naturally home on those sites. Often such linkages are made through gene fusions between the active and the targeting proteins.

Polyethylene glycol spacer or linker units have been used to create antibody targeted superantigens after the date of the instant invention. A monoclonal antibody reactive to colon carcinoma cells was attached to the bacterial superantigen staphylococcal enterotoxin. Rather than being designed to exploit the benefits associated with the other bivalent complexes (e.g., higher molecular weight; synergistic effects of bivalency) these complexes are designed to target superantigens to specific locations. The pegylation process described to form these targeted superantigens creates a complex containing a large mixture of materials. The coupling of the antibody and the superantigen was accomplished by the use of N-succinimidyl 3-(2-pyridyldithio) proprionate and a 24-atom-long PEG-based hydrophilic space. According to this procedure 7 to 18 spacers were attached to each antibody unit and one or two lysines on each of the super antigens were reacted. Dohlsten et al., Proc.

Natl. Acad. Sci. USA vol. 88, pg. 9287 (October, 1991). Using this procedure it would be impossible to isolate a single species in order to optimize the product or process.

Two groups of proteinaceous materials having significant applications for the treatment of a wide variety of medical indications are Tumor Necrosis Factor (TNF) inhibitors and Interleukin-1 receptor antagonists (Il-1ra). These materials have been shown to have beneficial effects in the treatment of TNF and IL-1 mediated diseases respectively. Among the indications that have been identified as being either TNF mediated or IL-1 mediated, are Adult Respiratory Distress Syndrome, Pulmonary Fibrosis, Rheumatoid Arthritis, Inflammatory Bowel Disease and Septic Shock.

Copending U.S. Patent Application Serial No. 555,274, filed July 19, 1990, specifically incorporated herein by reference, describes a class of naturally occurring proteinaceous TNF inhibitors and a method for manufacturing a substantial quantity of the same with a high degree of purity. In particular, the aforementioned application describes in detail two subsets of TNF inhibitors referred to as 30kDa TNF inhibitor and 40kDa TNF inhibitor. In addition to the full-length 40 kDa TNF inhibitor protein, two truncated, yet biologically-active, forms of the 40 kDa TNF inhibitor have also been produced. These proteins, in which 51 and 53 carboxyl termini amino acids have been removed from the full-length protein, are referred to respectively as 40 kDa TNF inhibitor Δ51 and 40 kDa TNF inhibitor Δ53.

Copending U.S. patent application Serial No. 07/506,522, filed April 6, 1990, specifically incorporated herein by reference, describes a preferred class of naturally occurring, proteinaceous Il-1 inhibitors and a method for manufacturing a substantial quantity of the same with a high degree of purity. In

particular, the application describes in detail three such interleukin-1 inhibitors which are interleukin-1 receptor antagonists (IL-1ra's), namely, IL-1raα, IL-1raβ, and Il-1rax.

Two additional classes of materials that are potentially useful for the treatment of a variety of medical indications are interleukin-2 inhibitors and complement inhibitors. Potential inhibitors of interleukin-2 include interleukin-2 receptors, the extracellular portion of interleukin-2 receptors, interleukin-2 receptor antagonists, antibodies that recognize interleukin-2, and fragments of any of such species that contain the IL-2 binding function. Potential inhibitors of the complement system include the receptor CR1, the extracellular portion of CR1, and the fragment of CR1 that contains the complement binding function.

Interleukin-2 receptor has been described and methods for its isolation have been disclosed in U.S. Patent No. 4,578,335 of Urdal et al. and U.S. Patent No. 4,816,565 of Honjo et al. The gene encoding Interleukin-2 receptor and methods for its recombinant production have also been disclosed. European Patent Application No. 89104023.0 of Taniguchi et al.; European Patent Application No. 90104246.6 of Taniguchi et al. See also, Honjo et al., Nature vol. 311, pg. 631 (1984); Taniguchi et al., Science vol. 244, pg. 551 (1989).

It could be assumed that to some extent the soluble extracellular domain of either interleukin-2 receptor will act as an inhibitor to the action of the cytokine interleukin-2. Interleukin-2 is one of the best characterized cytokines, known to play a pivotal role in the antigen-specific clonal proliferation of T lymphocytes. Interleukin-2 has also been shown to act on a variety of other cells in the immune system.

There are three discrete forms of the

interleukin-2 receptor, comprised of two distinct receptor molecules designated either as IL-2rα and IL2rβ.

The highest affinity IL-2 receptor is composed of two distinct IL-2 receptors. Both of these receptors have been cloned and characterized. The low affinity IL-2 receptor (IL-2rα) was cloned in 1984 and has been well characterized. Nikaido et al., Nature vol. 311, pg. 631 (1984). The extracellular domain of the molecule has a molecular weight of 24,825 and has two N-linked glycosylation sites. The molecule contains 11 cysteines, 10 of which are involved in intramolecular disulfide bonds. The putative IL-2 binding domains on the molecule have been mapped both by mutagenesis and epitope mapping. The intermediate affinity IL-2 receptor (Il-2rβ) was cloned in 1989 and has not been as completely characterized as IL-2rα. Hatakayama et al., Science vol. 244, pg. 551 (1989). The extracellular domain of IL-2rβ has a molecular weight of 24,693. The molecule contains 8 cysteines and 4 N-linked glycosylation sites. The disulfide bonding in the molecule is unknown. IL-2rβ has a cytoplasmic domain of 286 amino acids.

The disassociation constants (Kd's) for the IL-2 receptors have been determined. They are 10^{-8}M for IL-2rα, 10^{-9}M for IL-2rβ and 10^{-11}M for the high affinity receptor which consists of a complex of IL-2rα, IL-2rβ and IL-2. Current models indicate that the formation of the high affinity complex is formed first by IL-2 binding to IL-2rα and then to IL-2rβ. Ogura et al., Mol. Biol. Med. vol. 5, pg. 123 (1988).

An inhibitor of IL-2 may be valuable in the prevention of transplant rejection as well as autoimmune disorders. Currently, a monoclonal antibody against IL-2rα that prevents IL-2 binding is being tested in human renal transplantation. Hiesse et al., La Presse Mediocle vol. 20, pg. 2036 (1991). In a

study of 15 patients, the antibody, in combination with immunosuppressants, has been shown to be as effective in preventing allograft rejection as a control group getting higher doses of immunosuppressants. High levels of circulating soluble IL-2rα have been detected in a number of diseases, some infections, as well as transplantation and rejection. This suggests involvement of IL-2 in these diseases.

CR1 is a protein also referred to as the C3b/C4b receptor. CR1 is present on erythrocytes and a variety of other cell types, and specifically binds C3b, C4b, and iC3b. CR1 can also inhibit the classical and alternate pathway C3/C5 convertases and act as a cofactor for the cleavage of C3b and C4b by factor 1. Fearon et al., Proc. Natl. Acad. Sci. USA vol. 75, pg. 5867 (1979). CR1 is a glycoprotein composed of a single polypeptide chain, and there are four allotypic forms. It is known that CR1 contains repetitive coding sequences, and this fact is used to explain the existence of multiple allotypes. Krickstein et al. Complement vol. 2, pg. 44 (Abst.) (1985).

The diminished expression of CR1 on erythocytes has been associated with systemic lupus erythematosus and CR1 number has also been found to correlate inversely with serum level of immune complexes. The CR1 protein, the CR1 gene and methods for the production of CR1 are described in WO 91/05047 and WO 89/09220 of Fearon et al. As described above, dimeric species containing CR1 and portions of an antibody have also been disclosed. WO 91/16437 of Hebell et al.

SUMMARY OF THE INVENTION

This invention relates to a method for modifying polypeptides and the resulting modified polypeptides.

This invention includes substantially purified compounds comprised of the formula R_1-X-R_2 wherein R_1

and R_2 are biologically active groups and X is a non-peptidic polymeric spacer. R_1 and R_2 may be the same or different groups, and at least one of the R_1 and R_2 is polypeptidic. In the preferred embodiments, R_1 and R_2 are selected from the group consisting of interleukin-1 receptor antagonist; 30kDa tumor necrosis factor inhibitor; interleukin-2 receptor and CR1, and X is selected from the group consisting of polyethylene glycol, polyoxyethylated glycerol, dextran, colonic acids, poly β-amino acids and carbohydrate polymers. Also included are pharmaceutical compositions comprised of such substantially purified compounds in a pharmaceutically acceptable carrier. Further included are methods of treating patients in need thereof with such pharmaceutical compositions. The compounds of the formula R_1-X-R_2, as depicted in Figure 19, are referred to as "dumbbells".

This invention also includes a method for the preparation of substantially purified therapeutically valuable compounds comprised of the formula R_1-X-R_2 comprising reacting a non-peptidic polymeric group having at least two reactive groups capable of forming covalent bonds with the biologically active group R; and isolating said compound.

In an alternate embodiment, this invention includes a method for the preparation of substantially purified therapeutically valuable compounds, comprised of the formula R_1-X-R_2, wherein R_1 and R_2 are different, comprised of: reacting a non-peptidic polymeric group capable of forming covalent bonds when reacted with the biologically active group R_1 to form a complex R_1-X; reacting complex R_1-X with the biologically active group R_2 to form said compound; and isolating and purifying said compound.

In one embodiment, this invention relates to the site-specific pegylation of TNF inhibitor and IL-1 inhibitor proteins.

In order to maintain site-specifity of pegylation, pegylating reagents are selected that will react almost exclusively with the free -SH groups of cysteine residues of the polypeptides. An example of a pegylation reagent that covalently binds almost exclusively to the -SH groups of cysteine is 0-(2-maleimido ethyl)-0' methlypolyethylene glycol.

Site specific pegylation may be done at either naturally occurring "free" cysteine residues of a given polypeptide, or at free cysteines contained on muteins of the naturally-occurring polypeptides. Cysteines may either be added to or inserted into the amino acid sequence of the naturally occurring polypeptide, or substituted for other amino acid residues at selected locations.

In one embodiment of this invention, the polypeptides that are to be pegylated are produced via recombinant DNA technology from a bacterial host cell. In most cases the bacterially expressed polypeptide must be refolded to obtain biological activity prior to the pegylation step. In certain applications of this invention, the native polypeptide does not contain any free cysteine residues, but an altered polypeptide is produced to contain at least one free cysteine in the biologically active polypeptide. According to this method, the refolding of the bacterially expressed polypeptide is facilitated by the addition, in turn, of a sulfhydryl containing compound such as cysteine and a disulfide containing compound such as cystine. After refolding and purification, the polypeptide is treated with a limited amount of a mild reducing agent such as dithiothreitol ("DTT") to regenerate the sulfhydryl group of the novel cysteine residue of the altered polypeptide. Following dialysis under conditions designed to prevent oxidation, the polypeptide may be reacted with a cysteine specific pegylation agent to site specifically form a covalently modified

polypeptide.

Preferred pegylated polypeptides of the present invention are site-specifically pegylated TNF-inhibitors and IL-1 inhibitors. More specifically, this invention describes pegylated 30kDa TNF inhibitor and pegylated IL-1 receptor antagonist. Most preferred pegylated TNF inhibitors include 30kDa TNF inhibitor wherein the asparagine amino acid residue at position 105 of the native human protein is changed to cysteine using in vitro mutagenesis and pegylation has occurred at the free cysteine at position 105. Other pegylated derivatives of mutated 30kDa TNF inhibitors include mutations where cysteine has been added at positions 1, 14, 111 and 161. In addition to the singly pegylated muteins, any and all combinations of the various mutations may be included within a single mutein to create altered 30kDa TNF with more than one free cysteine residue capable of being pegylated.

The most preferred pegylated IL-1ra includes native or naturally occurring IL-1ra, which includes four free cysteines. Mono pegylation of the native IL-1ra yields site-specific pegylation at cysteine position 116. Other pegylated derivatives of mutated IL-1ra include muteins having cysteine added at the amino terminus of the polypeptide, cysteine added at positions 6, 8, 9, 84, or 141, and the replacement of the cysteine at position 116 with serine. In addition to the singly pegylated muteins, any and all combinations of the various mutations may be included to create altered IL-1ra with more than one free cysteine capable of being pegylated.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the invention, including illustrative examples of the practice of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amino acid sequence of native IL-1ra.

Figure 2 depicts the amino acid sequence of native 30kDa TNF inhibitor.

Figure 3 shows the Coomassie SDS-PAGE of unpegylated and pegylated forms of IL-1ra and the mutein c84s116 IL-1ra. Lanes 2, 3, 5 and 6 contain pegylation reaction mixes. Lanes 1 and 4 are the unmodified proteins:

Lane 1 - IL-1ra

Lane 2 - $mPEG^{*}_{5000}$ IL-1ra

Lane 3 - $mPEG^{*}_{8500}$ IL-1ra

Lane 4 - c84s116 IL-1ra

Lane 5 - $mPEG^{*}_{5000}$ c84s116 IL-1ra

Lane 6 - $mPEG^{*}_{8500}$ c84s116 IL-1ra

Figure 4 depicts the mono S ion exchange chromatography of: Chromatogram A, the pegylation reaction mixture of $mPEG_{5000}^{*}$ IL-1ra, peak 1 is the modified and peak 2 is the unmodified Il-1ra; and Chromatogram B, shows the purified $mPEG_{5000}^{*}$ IL-1ra.

Figure 5 depicts a size exclusion chromatogram showing the elution profile of several size standards, and $mPEG_{8500}^{*}$ IL-1ra (fraction 7) and Il-1ra (fraction 13).

Figure 6 depicts the reverse phase HPLC fractionation of tryptic digest of alkylated $mPEG_{5000}^{*}$ IL-1ra reacted with tritiated iodoacetic acid to label free cysteines. Separation was performed on a Brownlee C8 (2.1 x 220mm) column at ambient temperature and a flow rate of 1000μL/min with a linear gradient. Solvent A was 0.1% TFA in water and solvent B was 0.085% TFA in 80% acetonitrile and 20% H_2O.

Figure 7 depicts the reverse phase HPLC fractionation of chymotryptic digest of peptide 18 in figure 6. Conditions were identical to those in Figure 6. Peptides 5 and 8 contained tritium counts and

peptide 5 had the amino acid sequence LCTAMEADQPVSL. The cysteine was identified as the carboxymethylcysteine derivative. This cycle was the only one containing counts above background. The amino acid sequence of peptide 8 began with serine 103 of Il-1ra. Redigestion of this peptide with chymotrypsin permitted fractionation of all tritium counts from the peptide.

Figure 8 depicts the plasma IL-1ra concentration versus time profiles of mature IL-1ra, pegylated IL-1ra, and several pegylated muteins of IL-1ra.

Figure 9 shows the SDS-PAGE gel showing c105 30kDa TNF inhibitor and mPEG, and the separation of unreacted 30kDa TNF inhibitor from mPEG c105 30kDa TNF inhibitor by size exclusion chromatography.

Figure 10 shows a plot containing intravenous plasma IL-1ra concentration versus time curves for a large number of singly PEGylated IL-1ra species, doubly PEGylated IL-1ra species, and IL-1ra PEG dumbbell species.

Figure 11 shows a plot containing subcutaneous plasma IL-1ra concentration versus time curves for a number of IL-1ra species as in Figure 10.

Figure 12 shows a plot of plasma IL-6 levels versus time after the injection of mice with hrTNF.

Figure 13 compares IL-6 levels induced in mice by five ratios of c105 30kDa TNF inhibitor to TNF (A) and five ratios of c105 30kDa TNF inhibitor PEG_{2000}db to TNF (B).

Figure 14 depicts plasma IL-6 levels induced in mice by TNF alone and one to one ratios of TNF to c105 30kDa TNF inhibitor PEG_{3500} and $PEG_{10,000}$ dumbbells.

Figure 15 depicts percent neutrophils induced by varying ratios of TNF to c105 30kDa TNF inhibitor (A), c105 30kDa TNF inhibitor PEG_{3500}db (B); c105 30kDa TNF inhibitor $PEG_{10,000}$db (C); and c105 30kDa TNF

inhibitor $PEG_{20,000}$db (D).

Figure 16 shows a plot containing intravenous plasma 30kDa TNF inhibitor concentration versus time curves for native 30kDa TNF inhibitor, c105 30kDa TNF inhibitor PEG_{8500}, and $PEG_{10,000}$ and 30kDa TNF inhibitor PEG_{3500}, $PEG_{10,000}$ and $PEG_{20,000}$ dumbbells.

Figure 17 shows a plot containing subcutaneous plasma 30kDa TNF inhibitor concentration versus time curves for a number of 30kDa TNF inhibitor species as in Figure 16.

Figure 18 depicts the solubility of 3 solutions of native IL-1ra and c84 IL-1ra PEG_{8500} by plotting O.D. 405 versus time.

Figure 19 depicts the basic structure of compounds of this invention having the general formula R_1-X-R_2 that are referred to as dumbbell compounds.

DETAILED DESCRIPTION OF THE INVENTION

This invention involves the selective modification of pharmaceutically useful polypeptides, in particular, Tumor Necrosis Factor ("TNF") inhibitors and interleukin-1 ("IL-1") inhibitors. More specifically this invention describes the selective modification of 30kDa TNF inhibitor and IL-1 receptor antagonist ("IL-1ra"). The selective modifications serve to both enhance the pharmacokinetic properties of the polypeptides as well as to provide homogenous compositions for human therapeutic use.

Additional polypeptides that may be selectively modified according to the procedures of this invention include interleukin-2 receptors ("IL-2r") and CR1. All references to interleukin-2 receptor shall be construed to include both α and β chains of IL-2r unless stated otherwise.

In the preferred embodiments of the invention the modified polypeptides and DNA sequences are human.

However, to the extent that there is sufficient homology between animal DNA and peptide sequences to the human forms, they would be included within the scope of this invention.

In one embodiment, the method of modification of the present invention includes covalently bonding long chain polymers to the polypeptides of interest in a site specific manner. The selected polypeptides may be the native or naturally occurring polypeptides of interest, or they may be biologically active muteins of the polypeptides that have been produced to enhance the modification process described herein. The method of the invention includes the selection, production and screening of desired muteins that will meet the objectives of this invention. In other embodiments of this invention the method for modifying polypeptides requires merely that the modification be made so that the resulting product be available in substantially purified form as that term is defined herein.

In certain embodiments, the modified polypeptides of the present invention will be bonded to long chain polymers at specific positions of the amino acid sequence. The modified polypeptides of the present invention will retain a substantial portion of their biological activity. In the preferred embodiments, the modified polypeptides will retain at least one tenth of the biological activity of the native polypeptide in a receptor binding assay. In a more preferred embodiment, the modified polypeptide will retain at least one fifth of the biological activity of the native polypeptide, and in the most preferred embodiment at least one fourth of the activity will be retained. In addition, the modified polypeptide will serve to improve the pharmacokinetic performance of the native polypeptide in at least one of the following areas:

1) increasing the apparent molecular weight

of the native polypeptide and, hence, reducing the clearance rate following subcutaneous or systemic administration;

2) increasing the solubility of the native polypeptide in aqueous solutions; or

3) reducing the antigenicity of the native polypeptide.

In many embodiments of the invention, each of these objectives will be accomplished. In the preferred embodiments of the invention, the long chain polymer will be polyethylene glycol or monomethoxy polyethylene glycol. A polyethylene glycol unit will be referred to herein as PEG and a monomethoxy polyethylene glycol unit will be referred to as mPEG. The approximate molecular weight of the polymeric unit will be given in subscripts. For example, a monomethoxy polyethylene glycol unit of approximate molecular weight of 5,000 will be depicted as $mPEG_{5000}$ or PEG_{5000}. Other long chain polymers included within the scope of this invention are polypropylene glycol ("PPG"), polyoxyethylated glycerol ("POG"), dextran, colonic acids or other carbohydrate-based polymers and polymers of β-amino acids and biotin derivatives.

In an alternate embodiment of the present invention, the long chain polymer unit is dihydroxy polyethylene glycol, or $HO-(CH_2CH_2O)_n-H$. When activated to bind covalently with polypeptides or other biologically active compounds as described below, the dihydroxy material will contain two reactive sites.

In the preferred embodiments of the present invention the long chain polymer units are bonded to the polypeptide via covalent attachment to the sulfhydryl group (-SH) of a cysteine residue. To obtain selectivity of reaction and homologous reaction mixtures, it is useful to utilize functionalized polymer units that will react specifically with sulfhydryl groups. The functional or reactive group

attached to the long chain polymer is referred to herein as the activating group. Activating groups include the maleimide group, sulfhydryl group, thiol, triflate, tresylate, aziridine, oxirane and 5-pyridyl. The preferred activating groups are maleimides.

Activated dihydroxy polyethylene glycols, because of the physical separation between the ends of the polymeric chain, are nearly equally reactive at each end of the molecule. By appropriate selection of reaction conditions and polypeptides, the activated dihydroxy polyethylene glycols--or any other multi-activated long chain polymer unit--will react with polypeptides to form "dumbbell" shaped complexes where two polypeptides are joined by a long chain polymeric unit.

By utilizing the different rates of reaction that would be found between the activated polymeric linked group and different cysteine-containing polypeptides and by the kinetics of the reactions, it is easily within the skill of those in the art to also produce dumbbell complexes where substantially purified compounds can be formed comprising two different polypeptide groups, or comprising a single polypeptide group and a different biologically active group. Examples of such heterodumbbell compounds are given below.

The extent and availability for reaction of cysteines varies dramatically from polypeptide to polypeptide. Therefore, in the biologically-active form many polypeptides do not have "free" cysteines, or cysteines not bound to another cysteine. In addition, the existence of "free" cysteines does not mean that cysteines are accessible for binding to reactive reagents. Since the modification usually occurs on the active or three dimensionally folded polypeptide, little or no reaction will occur when a free cysteine is found within the "interior" of the folded structure.

A further constraint when modifying polypeptides is the potential effect the modification may have on the active site of the polypeptide. The modification of a cysteine having a certain proximal relationship to the active site may effectively deactivate the polypeptide. Even when a great deal is known about the selected polypeptide, it is difficult, if not impossible, to accurately predict which cysteine residues may be effectively modified.

The same factors also exist when mutated polypeptides are produced that contain additional cysteine residues. When the polypeptide is recombinantly produced via bacterial expression, the non-native cysteines may interfere with the proper refolding of the polypeptide. In addition, the cysteine must be accessible to the pegylating reagent, and the pegylated cysteine must not significantly interfere with the active site of the polypeptide.

The selection of potential sites within a given polypeptide for the introduction of a non-native cysteine can be influenced based on various sources of information. For example, glycosylation sites may be a good site for a mutation to include a free cysteine. To the extent that information is known about the binding or active site of the polypeptide, that information can also be used to select potential muteins. The addition or substitution of a cysteine residue at the amino terminus or carboxyl terminus of the polypeptide is also a likely prospect because of its location. And finally, the mutation of lysine residues to cysteine may be considered based on the assumption that lysines will generally be found on the surface of the biologically active polypeptide.

Although a variety of potential muteins can be selected for a given polypeptide that may meet the desired characteristics, it is only through the synthesis, pegylation and testing of such altered

muteins that it will be known which will meet the objectives of the present invention. In light of this invention and the general skill and knowledge of those skilled in the art, such synthesis, pegylation and testing can be performed without undue experimentation. It should be noted, that even if the pegylation of a polypeptide acts to reduce the biological activity of a polypeptide to a certain extent, the improvement in the pharmacokinetic performance of the polypeptide may greatly increase the value of the native polypeptide in various therapeutic applications.

Upon selection of target muteins, the preferred method for the production of the muteins is by recombinantly expressing the gene coding for the mutein. Assuming that the gene coding for the native polypeptide is known, the altered gene may be created either by standard site specific mutagenesis procedures on the native gene, or by the construction of the altered gene by standard gene synthesis procedures. These techniques are well known to those of ordinary skill in the art.

The gene coding for the target mutein may be expressed in a variety of expression systems, including animal, insect and bacterial systems. To the extent that expression systems have been perfected for the expression of the native polypeptides, the same systems may be used for the target muteins. In the preferred embodiment of the present invention, the genes coding for the target muteins are produced by site specific mutagenesis of the native gene, and the gene encoding the mutein is expressed from a bacterial expression system. The gene encoding native IL-1ra and a method for expressing said gene in E. Coli is described in detail in United States Patent No. 5,075,222 of Hannum et al., issued December 24, 1991. The gene encoding native 30kDa TNF Inhibitor and a method for expressing said gene in E. Coli is described in detail in United

States Patent Application Serial Number 07/555,274 filed July 19, 1990. Each of these applications is incorporated herein by this reference.

The muteins and pegylated materials of the present invention include allelic variations in the protein sequence (sequence variations due to natural variability from individual to individual) and substantially equivalent proteins. "Substantially equivalent," as used throughout the specification and claims is defined to mean possessing a very high degree of amino acid residue homology (See generally, M. Dayhoff, Atlas of Protein Sequence and Structure, vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by references) as well as possessing comparable biological activity. Also included within the scope of this invention are muteins and pegylated polypeptides that are partially truncated versions of the native polypeptide.

In one preferred embodiment of the method of the present invention when the target mutein is produced via recombinant DNA technology in a bacterial expression system, the following steps are performed:

1) The gene coding for the target mutein is created by site directed mutagenesis of the gene coding for the native polypeptide;

2) The gene coding for the target mutein is expressed in a bacterial expression system;

3) The target mutein is isolated from the bacteria and purified;

4) The target mutein is refolded in the presence of cysteine or another sulphydryl containing compound;

5) The refolded target mutein is isolated and purified;

6) The purified and refolded target mutein is treated with a mild reducing agent;

7) The reaction mixture is dialyzed in the absence of oxygen; and

8) The dialyzed reaction mixture is treated with a long chain polymer containing an activating group.

In the preferred embodiment for the production of pegylated muteins of 30kDa TNF inhibitor, the mild reducing agent is dithiothreitol ("DTT"). In an alternate embodiment, the modification may occur prior to the refolding of the expressed protein or mutein.

In the preferred embodiment of the present invention, the pegylated muteins and pegylated native polypeptides may be purified and formulated into pharmaceutical compositions by conventional methods. In an alternate embodiment, the purified muteins may also be formulated into pharmaceutical compositions.

The pegylated polypeptides of the present invention formed by the reaction of a deactivated long chain polymer unit have additional beneficial properties. These "dumbbell" shaped molecules can contain two of the polypeptides of interest attached by a single polymer unit. This structure imposes a certain amount of linearity to the polymeric molecule and reduces some of the steric hinderance inherent in the use of large hydrophilic polymers such as polyethylene glycol. The goal of obtaining molecules with increased apparent molecular weight is achieved while retaining high biological activity. Included specifically within the scope of this invention are bidentate molecules where two IL-1ra molecules or two TNF inhibitor molecules are covalently attached to a single polymeric chain, or where two different polypeptides are attached to a single polymeric chain, i.e., a single bidentate molecule containing both a TNF inhibitor and a IL-1ra moiety.

Native IL-1ra (figure 1) and various muteins of IL-1ra have been pegylated according to the present

invention. Pegylation of wild type IL-1ra at free sulphydryl groups, by the methods described in the examples below, results in the addition of mPEG at the cysteine residue at position 116 of IL-1ra (c116). The other three cysteines are not accessible for pegylation in the fully native molecule. To attach mPEG molecules at different sites of IL-1ra and to make mPEG conjugates having more than one mPEG, IL-1ra in which native amino acids in IL-1ra were replaced with a cysteine, or additional cysteines are added at the amino-terminus of the protein. To prepare conjugates in which residue 116 is not pegylated c116 has been changed to a serine in a number of the muteins. Below is a list of the muteins that have been generated for reaction with mPEG (the residue numbering is based on the sequence given in Figure 1; c referring to cysteine and s referring to serine):

c0s116	c0c116
c84s116	c84c116
c6s116	c6c116
c8s116	c8c116
c9s116	c9c116
c141s116	c141c116

Native 30kDa TNF inhibitor (figure 2) does not contain any free cysteine residues. The following muteins of 30kDa TNF inhibitor have been prepared (the residue numbering is based on the sequence given in Figure 2; c referring to cysteine):

c105 30kDa TNF inhibitor
c1 30kDa TNF inhibitor
c14 30kDa TNF inhibitor
c111 30kDa TNF inhibitor
c161 30kDa TNF inhibitor

Included within the scope of this invention is an entire class of compounds, as depicted in Figure 19, that can be represented by the formula R_1-X-R_2 wherein R_1 and R_2 are biologically active groups and at least

one of R_1 and R_2 is polypeptidic, and X is a non-peptidic polymeric spacer or linker group. R_1 and R_2 may be the same group or different. Where R_1 and R_2 are different groups, both R_1 and R_2 may be polypeptidic, or R_1 may be polypeptidic and R_2 may be any biologically active group. The compounds having this structure, which have been referred to as "dumbbell" compounds, are characterized by being substantially purified. "Substantially purified" in this context is defined as being a homogenous composition.

A homogenous composition consists of one molecule of the linker X and one molecule of R_1 and one molecule of R_2. A homogenous composition includes, but does not require, that the biologically active groups R_1 and R_2 be attached to the linker at the exact same location on the groups in each molecule of the compound. In certain embodiments of the invention, the biologically active groups are attached site specifically to the linker. For example, in the compound c105 30kDa TNF inhibitor PEG_{3000}db, two c105 30kDa TNF inhibitor groups are attached at the 105 cysteine residue to the PEG_{3000} linker.

When referring to a "homogenous composition" it is to be understood that on a molecule-by-molecule basis, the dumbbell compound is also not necessarily homogenous with respect to the exact length of the spacer group. It is understood by those skilled in the art that any production process that utilizes a given weight range of PEG or other higher molecular weight polymer begins with a solution that contains an "average" molecular weight. Therefore, when a bis-reactive PEG unit is reacted with a polypeptidic group, the PEG unit is by definition polydisperse, and the resultant dumbbell compound is heterogenous to the extent that the length of the linker is subject to the variation known to exist by those skilled in the art.

In summary, "substantially purified" in this context refers to materials that are substantially free from compounds: 1) that deviate in the composition of R_1 or R_2; or 2) that are linked together by more than one linker X.

R_1 and R_2 are defined as being biologically active groups. Biologically active groups include any compound that can induce a biological effect on interact with a natural biological molecule. Biologically active groups include proteins, polypeptides, steroids, carbohydrates, organic species such as heparin, metal containing agents, vitamins, or any other biologically active species. At least one of the groups R_1 and R_2 is polypeptidic. In the preferred embodiment, both R_1 and R_2 are polypeptidic.

Polypeptidic is defined as any compound that is substantially proteinaceous in nature. However, a polypeptidic group may contain some non-peptidic elements. For example, glycosylated polypeptides or synthetically modified proteins are included within the definition.

The biologically active groups R_1 and R_2 include binding groups and targeting groups. Binding groups are defined by their affinity for a given biological ligand. Targeting groups are defined by their ability to direct the location of a complex within a biological system. R_1 and R_2 may have affinity for the same ligand, in which case the dumbbell may have enhanced affinity to that ligand. R_1 and R_2 may have an affinity for different ligands, wherein R_1 serves to target the complex into a location where the ligand for R_2 predominates.

Preferred polypeptidic groups are receptors, the extracellular portions of receptors, cell surface molecules, and extracellular matrix molecules, binding proteins, and receptor antagonists. Included among the polypeptidic groups that may be used as R_1 or R_2 are

the following polypeptides and any fragment thereof: IL-1 receptor antagonist, 30kDa TNF inhibitor, 40kDa TNF inhibitor, Il-2 receptor, CR1 (all references to CR1 include any single or combination of consensus repeat sequences of CR1), PDGF receptor, IL-2, MCSF receptor, EGF receptor, IL-5 receptor, IL-3 receptor, GMCSF receptor, T-cell receptor, HLA-I, HLA-II, NGF receptor, IgG (V_H, V_l), CD40, CD27, IL-6 receptor, Integrins CR3, VLA_4, ICAM, and VCAM, CR2, GMP140 Lec domain, Laminin binding protein, Laminin fragments, Mannose binding protein, exon 6 peptide of PDGF, and proteases (with 2 catalytic domains or a target domain and a catalytic domain). All references to receptors includes all forms of the receptor whenever more than a single form exists. In the preferred embodiments, the groups R_1 and R_2 are selected from the group consisting of IL-1 receptor antagonist, 30kDa TNF inhibitor, CR1, and IL-2 receptor (both the α and β chains).

In a preferred embodiment, the non-peptidic polymeric spacer X may be further defined as follows: $X = -Y_1-(Z)_n-Y_2-$, wherein Y_1 and Y_2 represent the residue of activating groups that react with R_1 and R_2 to link the spacer to the groups R_1 and R_2, and $(Z)_n$ represents the base polymeric group. According to the present invention n is greater than 6 and preferably is greater than 10.

Non-peptidic is defined as a polymeric group that is substantially not peptidic in nature. The inclusion of less than 50% by weight of α-amino acid residue as part of Y_1, Y_2 and Z would be considered substantially non-peptidic in nature and would be considered non-peptidic. In the preferred embodiment, the non-peptidic spacer X is non-immunogenic, and biologically inert and hydrophilic. In addition, the preferred linkers are capable of conveying desirable properties to the biologically active polypeptidic groups -- such as reduced immunogencity, increased

solubility, or reduced clearance rate from the body -- without significantly reducing the affinity of a given R_1 or R_2 group to its ligand. In the most preferred embodiments, the compound R_1-X-R_2 (wherein R_1=R_2 and R_1 and R_2 are binding groups) has an affinity for its ligand that exceeds the affinity that the non-derivitized binding group has to the ligand. For example, sub-stantially purified c105 30kDa TNF inhibitor PEG_{3400}db has an inhibitor activity for TNF that is greater than 20 times the inhibitor activity that c105 30kDa TNF inhibitor has for TNF.

The activating groups Y_1 and Y_2 that are part of the polymeric spacer X may be comprised of any of the activating groups as discussed above, including the maleimide group, sulfhydryl group, thiol, triflate, tresylate, aziridine, oxirane, and 5-pyridyl. The preferred activating groups are maleimides.

The polymeric group $(Z)_n$ is preferably selected from the group consisting of polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dextran, poly β-amino acids, colonic acids or other carbohydrate polymers and polymers of biotin derivatives. In the preferred embodiments, the polymeric group is polyethylene glycol. Any non-peptidic polymeric group that would serve the functions as described herein would also be included within the scope of this invention.

One of the advantages of the present invention is the ability to vary the distance between the groups R_1 and R_2 by varying the length of the polymeric group linking the two binding groups. Although not limited by theory, it is proposed that the increase in biological activity seen for the multimeric compounds of this invention may be attributed to the multimeric nature of the cell receptors and ligands <u>in vivo</u>. For this reason, the optimal distance between the units R_1 and R_2 (which would be generally directly proportional

to the length of the polymeric unit $(Z)_n$) may be easily determined by one skilled in the art by varying the size of the spacer X.

In one embodiment of the present invention, the groups R_1 and R_2 are the same. However, in an alternate embodiment R_1 and R_2 are different species. Such compounds can be designed to create a heterodimer wherein both R_1 and R_2 act within the same general biological systems. For example, both IL-1 receptor antagonist and TNF inhibitors are believed to disrupt the inflammation cascade. The difunctional complexes may also be designed where R_1 or R_2 is a "targeting" species that "directs" the complex to a specific location by its binding affinity to a certain substrate, and the opposing binding group has a desired activity at the localized site.

An example of a heterodimer that has great potential for being a successful IL-2 inhibitor is one where R_1 is IL-2rα and R_2 is IL-2rβ. Such a heterodimer mimics the receptor complex that has the highest affinity for IL-2. See Example XVII. An additional heterodimer that can act as a complement inhibitor is the heterodimer where R_1 is the C3b binding domain from CR1 and R_2 is the C4b binding domain from CR1. See Example XVIII. In an additional heterodimer R_1 is the exon 6 peptide of PDGF and R_2 is IL-1ra. See Example XIX.

In the preferred embodiment of the invention, the procedures for producing the bifunctional R_1-X-R_2 complexes are essentially the same as those used for the site-selective reaction of polypeptides as described above. The synthesis of c105 30kDa TNF inhibitor PEG_{3400}db is described below in Example 13. A bis-reactive polymeric group is reacted with a cysteine-containing polypeptide, wherein the activating group on the bis-reactive polymeric group forms a thio-ether bond with the selected free cysteine residue. As described above, the cysteine may be a free cysteine

naturally-occurring on the polypeptidic group, or a non-native cysteine that has been added or substituted into the natural sequence.

The preferred bis-reactive polymeric compound of the present invention is α-(2-maleimido)ω-maleimido poly(oxyethylene) or bis-maleimido PEG. The synthesis of bis-maleimido PEG is described in Example 12. According to the preferred method, the bis-maleimido compound is prepared from bis-hydroxyl PEG via the bis-amino intermediate.

Several methods for the conversion of the terminal hydroxyls of PEG to the corresponding amino group have been reviewed by Harris et al., J. Polymer Sci. vol. 22, pg. 341 (1984); Harris, Rev. Macromol. Chem. vol. c25(3), pg. 325 (1985). This is accomplished by generating a reactive intermediate via either sulfonation, halogenation, or oxidation of the hydroxyl followed by displacement of the activated termini by a nucleophile.

Other practical alternatives to the synthesis of the bis-maleimide PEG given in Example 12 also exist. The reactive intermediate in the conversion of the hydroxyl to the amine may be the halogenated derivative (e.g. the α-(bromoethyl)-ω-bromopoly(oxyethylene) intermediate (Johannson, Biochim. et Biophy. vol. 222, pg. 381 (1970)) followed by direct substitution with ammonia, (Buckmann et al., Makromol. Chem. vol. 182, pg. 1379 (1981)) or the aldehyde intermediate (Harris, supra.) The bis-maleimide PEG is not the only sulfhydryl-specific reagent that may be used. Glass and coworkers have developed another method for the attachment of PEG to sulfhydryls. Glass et al., J. Biopolymers vol. 18, pg. 383 (1979). However, the reaction is reversible with thiols. Another method for attachment of PEG to cysteinyl sulhydryls is the bis-4-vinylpyridine PEG derivative.

Harris (supra) also reviews the synthesis of a variety of electrophilic derivatives of PEG that can be used as reagents to modify proteins. The reagents include chlorocarbonates, isocyanate, epoxide, succinimidyl succinate, cyanuric chloride, mixed anhydride, carbodiimides and sulfonates. The latter group includes tresylate, tosylate, and mesylates. Some of the reagents react selectively with amines (e.g., cyanuric chloride and carbodiimides) while others react with both sulhydryls and amines (e.g., epoxide and tresylates). Some of these reagents have been used to modify proteins and may result in varying degrees in loss of activity.

The preferred preparation of R_1-X-R_2 complexes where R_1 and R_2 are different requires a two step process where the bis-reactive polymeric group is reacted in series with R_1 and then R_2. The preparation of such heterodimers may be accomplished by those of ordinary skill in the art without undue experimentation. In some cases the intermediate R_1-X must first be isolated and purified prior to reaction with R_2, and in other circumstances an intermediate purification may not be necessary.

The extracellular domains of both IL-2rα and IL-2rβ may be cloned using PCR and cloned into a vector capable of directing expression in E. coli. The proteins may be refolded and purified from E. coli and their ability to inhibit IL-2 activity measured in bioassays. In vitro mutagenesis can be used to substitute native residues in the molecules with cysteine to allow for site directed attachment of PEG. Muteins of both IL-2rα and IL-2rβ may then be identified that allow for efficient attachment of PEG which do not lose activity when PEGylated. A PEG-linked heterodimer may be formed by first PEGylating IL-2rα in the presence of an excess of bis-maleimido PEG. The singly PEGylated IL-2rα may be purified and

IL-2rβ added to react with the active maleimide group and form the heterodimer. This molecule may be purified and its activity assessed. This molecule should mimic the high affinity IL-2 receptor found on cell surfaces.

A dumbbell complex where R_1 is IL-2 and R_2 is IL-2rβ should also be useful as a receptor antagonist of IL-2.

EXAMPLE I. SYNTHESIS OF POLYETHYLENE GLYCOLATING AGENTS

Three reagents are described to indicate the diverse means that may be used to derivatize polypeptides. See, Appendix to Example 1, for structures of Intermediates and reagents described below. All references provided below are specifically incorporated herein by this reference.

A. SYNTHESIS OF REAGENT 1: $mPEG_x$-ESTER-MALEIMIDE

The succinate ester derivative of the $mPEG_x$ (intermediate 1) was prepared as described by Wie et al. Int. Archs. Allergy App. Immun., vol. 64, pp. 84-99 (1981). The resulting product was weighed out and dissolved in a minimum of dry dioxane at 60°C. After the solution had cooled to ambient temperature, equimolar amounts of both tri-n-butylamine and isobutyl chloroformate were added. The reaction proceeded thirty minutes with stirring. During this time, a borate buffer, pH 8.8, was made by titrating a solution of 0.5 M boric acid with 1,6-hexanediamine. The solution containing the mixed anhydride was added dropwise to an aliquot of the borate buffer containing a 10-fold molar excess of 1,6-hexanediamine over the mixed anhydride. The reaction mixture was exhaustively dialyzed versus deionized water at 4°C and lyophilized. This polymer intermediate (intermediate 2) was reacted with a 2.5:1 molar excess of sulfosuccinimidyl 4-(N-

maleimiodethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemical Co., Rockford Ill.) in 50 mM sodium phosphate or HEPES buffer, pH 7.0, for two hours at room temperature. The resulting polymer was purified by size exclusion chromatography of the reaction mixture on Sephadex G-25 using 50 mM sodium phosphate (or HEPES) pH 7.0 for elution at 4°C. The maleimido-polymer (reagent 1) eluted at the void volume of the column and was detected by monitoring its absorbance at 260 nanometers. The reagent was used to alkylate polypeptides within one hour of its purification. Since the mPEG from this reaction can be removed by base hydrolysis, this reagent is useful for identifying the site of mPEG attachment to the protein.

B. SYNTHESIS OF REAGENT 2: $mPEG_x$-AMIDE MALEIMIDE

The $mPEG_x$-tosylate (intermediate 3) was prepared as described by Pillai et al. J. Org. Chem. vol. 45, pp. 5364-5370 (1980). The amount of sulfonated intermediate was estimated spectrophotometrically as described by Nilson and Mosbach, in Methods of Enzymology, vol. 104, pp. 56-69, Academic Pres. Inc., N.Y., N.Y. (1984). This intermediate was converted to the phthalimide derivative (intermediate 4) and subsequently reduced with hydrazine hydrate to the $mPEG_x$-NH_2 intermediate (intermediate 5) by the procedure of Pillai et al., supra. The amino group capacity in equivalents per gram of product was quantified by microtitration with hydrochloric acid. The $mPEG_x$-NH_2 was reacted with sulfo-SMCC in HEPES or phosphate buffer pH 7.2 at room temperature for two hours. The amount of the $mPEG_x$-amine to sulfo-SMCC was tested at molar ratios of 5:1 to 1:5.

To determine the optimal conditions the final reagent (reagent 2) was used in pegylation reactions and the quantity and quality of $mPEG_x$*IL-1ra (we will

use this designation for the pegylated product of IL-1ra reacted with reagent 2 and $mPEG_x$IL-1ra for pegylated IL-1ra from a reaction with reagent 3 described below) obtained from these reactions was assessed by SDS-polyacrylamide gel electrophoresis (PAGE). The optimal result was seen with a 1:1 ratio of SMCC to $mPEG_x$-NH_2. Higher proportions of sulfo-SMCC generated multiple higher molecular weight derivatives of IL-1ra on SDS-PAGE and multiple peaks on analytical ion exchange chromatography and lower proportions resulted in a reduced yield of pegylated protein. Reagent 2 was purified by size exclusion chromatography using G25 sephadex resin.

C. SYNTHESIS OF REAGENT 3: $mPEG_x$-MALEIMIDE

The $mPEG_x$-NH_2 (intermediate 5) can be modified further to yield a different maleimido-derivative (reagent 3). The latter was accomplished by reacting the $mPEG_x$-NH_2 with maleic anhydride via an adaptation of the procedure of Butler and Hartley, in Methods of Enzymology, vol. XXV pp. 191-199, Academic Press. Inc., N.Y., N.Y. (1972) and cyclizing this intermediate (intermediate 6) to the corresponding O-(2-maleimido ethyl)-O'-methylpolyethylene glycol using the method described by Wunsch et al., Biol. Chem. Hoppe-Seyler, vol. 366, pp. 53-61 (1985).

APPENDIX TO EXAMPLE I

SYNTHESIS OF REAGENT 1

Structures of starting material, intermediates and reagent from synthesis 1.

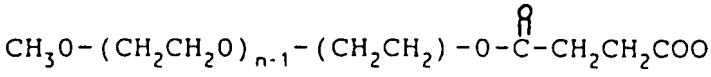
Starting material:

Generalized formula for monomethoxypolyethylene glycol (mPEGx):

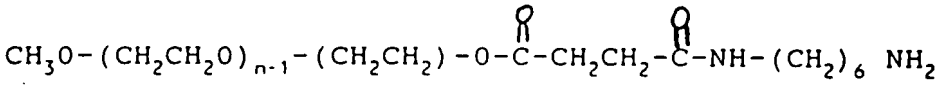
$$CH_3O-(CH_2CH_2O)_n-H$$

where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxythylene groups.

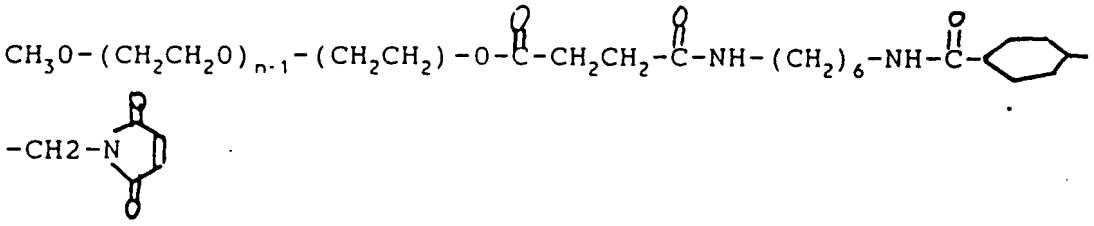
Intermediate 1:



Intermediate 2:



Reagent 1:

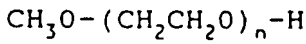


SYNTHESIS OF REAGENT 2

Structures of starting material, intermediates and reagent from synthesis 2.

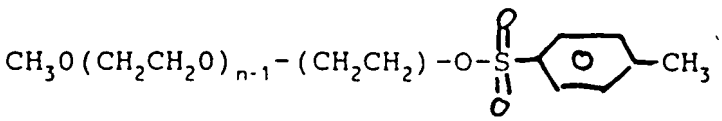
Starting material:

Generalized formula for monomethoxypolyethylene glycol (mPEGx):

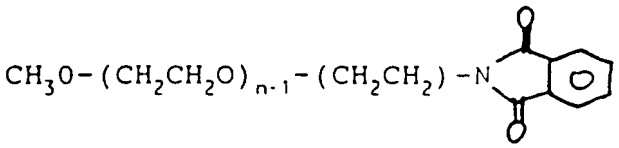


where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxyethylene groups.

Intermediate 3:



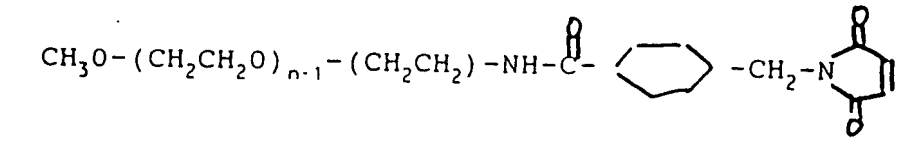
Intermediate 4:



Intermediate 5 (mPEGx-NH_2):

$CH_3O-(CH_2CH_2O)_{n-1}-(CH_2CH_2)-NH_2$

Reagent 2:



SYNTHESIS OF REAGENT 3

Structures of starting material, intermediates and reagent from synthesis 3.

Starting material:

Intermediate 5 ($mPEG_x$-NH2):

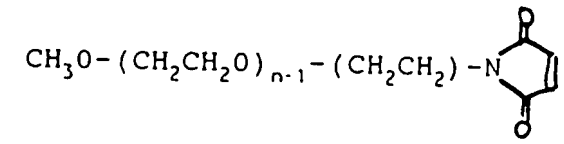
$CH_3O-(CH_2CH_2O)_{n-1}-(CH_2CH_2)-NH_2$

Intermediate 6:

$CH_3O-(CH_2CH_2O)_{n-1}-(CH_2CH_2)-NH-C-CH=CH-COOH$

Reagent 3:

O-(2-maleimidoethyl)-O'-methyl polyethylene glycol



EXAMPLE II. PREPARATION OF PEGYLATED NATIVE IL-1ra

Various parameters were tested in optimizing the pegylation reaction of native IL-1ra with successful pegylation assayed by visual inspection for a single tight band at 29 kilodaltons on Coomassie stained SDS-PAGE and a single sharp peak by analytical ion exchange chromatography. Unless otherwise stated, pegylation reactions were done at 1 mg/ml of native IL-1ra at room temperature in HEPES buffer pH 7.2 with a mPEG reagent to IL-1ra ratio of 2:1. The reagent used in these studies was mPEG-amido-maleimide (Reagent 2) and the product is referred to as $mPEG_x$*IL-1ra but the results are applicable to all three reagents.

A. TIME

Pegylation reactions at room temperature were analyzed from 0.5 to 24 hours. Conversion of the IL-1ra to the pegylated form is complete (80%-90%) in two to four hours and the total amount of mPEG*IL-1ra does not increase or decrease after longer periods of incubation. The quality of the mPEG*IL-1ra assayed by SDS-PAGE decreases at longer times due to the appearance of additional bands and smears at higher molecular weights on the stained gel.

B. TEMPERATURE

Pegylation reactions were incubated at temperatures of 4°, 25°, 37°, and 50°C and then analyzed at time points of 0.5, 1, 2, 4 and 17 hours. The reactions at 25° and 37° generated a large amount (about 50%-80%) of pegylated protein within one to two hours but those at 4°C and 50°C resulted in a much lower yield (10%-20%) even at the later time points. The quality of the mPEG*IL-1ra does not seem to change significantly with temperature.

C. PROTEIN CONCENTRATION

Pegylation reactions have been done with protein concentrations (native IL-1ra) between 50 ug/ml and 10 mg/ml. All of the concentrations tested worked well and there was no difference in the quality of the mPEG*IL-1ra.

D. pH

Native IL-1ra was pegylated under the reaction conditions stated above between pH 5.5 and 7.5. The quality of the mPEG*IL-1ra is slightly better by SDS-PAGE and ion exchange at a lower pH (5.5) but the percent conversion is the same.

E. mPEG-AMIDO-MALEIMIDE TO NATIVE IL-1ra RATIO

We tested ratios of between 0.5:1 to 20:1 of the mPEG-amido-maleimide to native IL-1ra. Ratios higher than about 2:1 result in efficient conversion to the pegylated form of IL-1ra (50%-90%). Ratios greater than 5:1, however, generate lower quality mPEG*IL-1ra by increasing the amount of extra high molecular weight bands on reduced SDS-PAGE and multiple peaks on ion exchange chromatography.

The optimal reaction conditions for both quantity of mPEG*IL-1ra obtained and quality of the material, within the parameters used, is a 2:1 mPEG-amido-maleimide/IL-1ra at 25°C for 2-4 hours using mPEG-amido-maleimide generated with a 1:1 ratio of Sulfo-SMCC to mPEG-amine. With these conditions 80-90% of the IL-1ra is converted to the pegylated form using reagent synthesized with either $mPEG_{5000}$ or $mPEG_{8500}$ as the starting material (Figure 3).

F. PREPARATION OF IL-1ra PEG DUMBBELLS

PEG dumbbell complexes containing IL-1ra are made according to the same procedures as other PEGylated IL-1ra species. A 2-4 molar excess of bis-maleimido PEG to IL-1ra in HEPES buffer at 7.0 is used. With IL-1ra, the species used may be the wild type molecule, which has a free and available cysteine residue, or a mutein prepared as described herein. The IL-1ra is at a concentration of 2-5 mg/ml. The reaction is incubated at ambient temperatures for 4 to 6 hours. The IL-1ra PEG dumbbell compounds are purified from the unPEGylated and singly PEGylated species by MonoS cation exchange at pH5.5 in 20-50mM MES buffer using a gradient from 0 to 1000 mM NaCl. Further purification may be achieved by size exclusion chromatography using a BioRad TSK 250 or Superdex 75 column, as described below.

EXAMPLE III. PURIFICATION OF PEGYLATED NATIVE IL-1ra

Purification of $mPEG_x$*IL-1ra can be achieved by cation exchange or size exclusion chromatography. These procedures are applicable to pegylated IL-1ra derived from all three reagents described above.

A. CATION EXCHANGE CHROMATOGRAPHY

The $mPEG_x$*IL-1ra can be purified using a MonoS (Pharmacia) column with 20mM MES buffer at pH 5.5. The proteins were eluted from the column using a salt gradient from 0 to 500mM NaCl in the same buffer. For example, unmodified IL-1ra elutes at 220mM NaCl, while the purity is assessed by various techniques including analytical ion exchange chromatography and SDS-PAGE. $mPEG_{5000}$ IL-ra elutes at 160mM (Figure 4).

B. SIZE EXCLUSION CHROMATOGRAPHY

The $mPEG_{5000}$*IL-1ra, which runs as about 52 kd, and $mPEG_{8500}$*IL-1ra, which runs as about 68 kd (based on column calibration with known size standards), can easily be separated from unmodified IL-1ra (17 kd) by size exclusion chromatography on a Superdex 75 (Pharmacia) column with standard chromatographic techniques (Figure 5).

EXAMPLE IV: CHARACTERIZATION OF THE $mPEG_x$*IL-1ra

Purified $mPEG_x$*IL-1ra gave a single symmetrical peak upon rechromatography on MonoS and appeared pure by both SDS-PAGE and size exclusion chromatography (Figure 3 and 4). A comparison of the tryptic maps of IL-1ra and $mPEG_{5000}$*IL-1ra showed one peak, corresponding to the peptide containing c116 and c122, absent from the conjugate map with the appearance of a new broad peak in this map. Subdigestion of this new peak with chymotrypsin and subsequent amino acid sequence analysis indicated that c116 had been pegylated under the conditions employed (Figure 6).

EXAMPLE V. PREPARATION OF IL-1ra MUTEINS

Mutagenesis was performed on single stranded DNA from the IL-1ra gene cloned into the bacteriophage M13. BioRad's Mutagene kit was used which uses the procedure described by Kunkel et al. Methods in Enzymology vol. 154, pp. 367-382 (1987). Briefly, single stranded DNA template was generated using an E. coli strain that contains the dut and ung mutations, resulting in template that contains uracil instead of thymidine. Mutagenic oligonucleotides between 20 and 30 base pairs in length were annealed to the template and the second strand was resynthesized using DNA polymerase and DNA ligase. The reaction mixtures were used to transform a wild type E. coli strain in which the uracil containing strand is degraded by the DNA repair mechanisms and the mutant strand is allowed to replicate. The mutant phage were screened and sequenced by standard techniques. The fragment containing the mutant gene was then subcloned into the expression vector pT5T (Eisenberg et al. Nature vol. 343, pp. 341-346, (1989)) and transformed into the T7 expression system strain (E. coli B121DE3). Other E. coli expression systems may also be used.

Expression clones were grown in Luria Broth supplemented with 15ug/ml tetracycline at 37°C. When the cultures reached an optical density of 0.8 at 600nm they were moved to 30° and IPTG was added to a final concentration of 1 mM to induce expression of the IL-1ra gene. Total accumulation of the IL-1ra protein was maximal after 4-6 hours and did not change significantly for up to 12 hours post induction.

EXAMPLE VI: PURIFICATION OF THE IL-1ra MUTEINS

Cell cultures induced as described above were harvested by centrifugation at 10000g for 10 min. The cells were resuspended in 30mM sodium acetate buffer pH 5.2 in 20-50 mls. Lysis was achieved by two passes

through the French Pressure cell at 18000 psi. The cell lysate was centrifuged at 10000g for 10 minutes. The soluble portion was loaded onto a S-Sepharose column and washed with the same buffer containing 75mM NaCl. The IL-1ra mutein eluted with buffer containing 200mM NaCl. The single pass over the ion exchange resin resulted in a product of sufficient purity (>95%) for pegylation studies. Further purification can be achieved using other ion exchange resins such as Q-Sepharose or MonoQ. This procedure was used for several of the IL-1ra muteins with equal success. In some cases it was necessary to vary the pH and/or NaCl concentrations slightly to purify muteins which have a small change in protein charge due the change in amino acid sequence. With these slight variations that would be easily manipulated by one of ordinary skill in the art, this procedure is generally applicable to all of the muteins studied.

EXAMPLE VII: IL-1ra MUTEIN PEGYLATION

In addition to the native IL-1ra, muteins c84s116, c84c116, c0s116 and c9s116 were pegylated. Employing the same conditions used for the native IL-1ra, the pegylated forms of c84s116 and c84c116 were produced and purified. Since c84c116 contains two reactive cysteines, pegylation results in a higher molecular weight protein at about 40 kd on SDS-PAGE. This protein can be purified by cation exchange or size exclusion chromatography and runs at the expected molecular weight of about 68 kd on the latter when using PEG_{5000}.

EXAMPLE VIII. EFFICACY OF THE mPEG*IL-1ra

The efficacy of the pegylated native IL-1ra molecules was tested by a standard competitive receptor binding assay using S^{35}-IL-1ra as the ligand. Mouse cells (EL4) containing the mouse type 1 IL-1 receptor

or hamster cells (CHO) expressing from a cloned gene the human type 1 receptor were used at 1 x 10^6 cells per well and 1 x 10^5 cells per well, respectively, in 96 well microliter dishes. S^{35}-IL-1ra with a specific activity of 4000 Ci/mmol was added to a final concentration of 150pM. Cold ligand was added in serial dilutions from 28 mM to 13 pM and allowed to incubate for 4 hours at 4°C. The cells were then filtered through a Milliliter filter plate (Millipore, .5 micron pore size Durapore filter), washed to remove nonspecifically bound counts, the filter removed and counted on an Ambis Radioanalytical Imaging System. Equilibrium dissociation constants (kDs) were calculated and used to compare the pegylated and unmodified forms of IL-1ra. Unmodified wild type IL-1ra and c84s116 have equal kD's for the type 1 mouse receptor of 150-300 pM in our assay. The kD for the IL-1ra pegylated form is about 400-800 pM and for pegylated c84s116, 500-1000 pM which is 2.5 and 3.5 fold higher than that of the unmodified protein respectively. The kDs for all but one (c6s116) of the unpegylated muteins are within 65-150% of the native protein, within the standard error of the assay. See Table 1.

TABLE 1

ANALYSIS OF PEGYLATED IL-1ra MOLECULES

	SIZE (kd)	RECEPTOR ASSAY
MUTEIN		
WILD TYPE	17.5	100
C84S116	17.5	98
C9S116	17.5	67
C6S116	17.5	37
C0S116	17.5	63
C84C116	17.5	95
PEG*IL-1ra		
SINGLE		
PEG_{5000}C116	50-60	34
PEG_{5000}C84S116	50-60	28
PEG_{8500}C116	70-80	30
PEG_{8500}C84S116	70-80	30
PEG_{8500}C0S116	ND	22
PEG_{8500}C9S116	ND	12
PEG_{12000}C116	78	20
DOUBLE		
PEG_{5000}C84C116	70-80	11
PEG_{8500}C84C116	150-200	4
PEG_{12000}C84C116	175	5
DUMBBELLS		
PEG_{3500}C116	55-65	49
PEG_{3500}C84	60	49
$PEG_{10,000}$C116	175-200	49
$PEG_{10,000}$C84	200	60
$PEG_{20,000}$C84	>200	24

Data are presented as a percent of the activity exhibited by unmodified Il-1ra. Standard deviations are within 10%.

EXAMPLE IX: PHARMACOKINETICS OF PEGYLATED NATIVE MUTEIN IL-1ra

The pharmacokinetic character of several pegylated native and mutein IL-1ra molecules was tested following intravenous injection of the molecules to rats. Native or pegylated IL-1ra was injected as an intravenous bolus dose (3 mg/kg). Serial blood samples

were drawn from the tail vein and assayed for native or pegylated IL-1ra by enzyme-linked immunosorbent assay (ELISA). The resulting plasma IL-1ra concentration vs. time profiles (Figure 8) illustrate that pegylation has a pronounced influence on the disappearance of IL-1ra from the plasma after intravenous injection. The declines in plasma IL-1ra and pegylated derivatives of IL-1ra are best described by three exponential components. The data indicate that pegylation prolongs the half-lives of these exponential components up to six-fold in the rat (Table 2). The half-lives of these exponential components increase as the size of the PEG molecule increases (Table 2). Additionally, there is evidence that the prolongation of the half-lives may be pegylation site-specific. Standard compartmental analysis was used to interpret the data of Figure 8.. The prolongation of half-lives may be explained based on accepted pharmacokinetic theory which states that the plasma half-life for a drug is inversely related to the plasma clearance for the drug and directly related to the apparent volume of distribution for the drug. Pharmacokinetic analysis of the disappearance of pegylated IL-1ras from the plasma indicate that the prolongation in half-life is inversely related to a decreased plasma clearance for the pegylated molecules, compared to native IL-1ra (Table 2). The decrease in plasma clearance is consistent with an anticipated size-related decrease in glomerular filtration of the pegylated molecules by the kidneys. Also, the prolongation of the half-lives by pegylation is directly related to an increase of the distribution (Vd steady-state, Table 2) of the pegylated molecule. The increase in distribution volume indicates greater penetration of the pegylated molecules into the extravascular pool. Through this mechanism pegylation improves therapy with IL-1ra by increasing the extent

to which the active molecules move from the systemic circulation into the extravascular compartment, a compartment in which IL-1 receptors are expected to be located. Because of the similarity between rats and humans in both clearance and distribution mechanisms for IL-1ra, it is apparent that pegylation will similarly improve the pharmacokinetic properties of IL-1ra in humans.

1. Additional intravenous pharmacokinetics for pegylated IL-1ra

The intravenous pharmacokinetics for eight additional pegylated IL-1ra muteins have been characterized using methods previously described. A plot containing intravenous plasma IL-1ra concentration vs. time curves for each of the molecules is attached (Figure 10). Review of all of the intravenous pharmacokinetic data (Table 3) indicates that as the size of the PEG (single or double) is increased, the plasma clearance decreases and hence the intravenous mean residence time and plasma IL-1ra disappearance half-lives increase. The site of pegylation is important in determining the extent to which the pegylation decreases the plasma clearance and prolongs the means residence time. The addition of two PEGs to IL-1ra prolongs the intravenous mean residence time fourteen-fold compared to wild type IL-1ra.

2. Subcutaneous pharmacokinetics for pegylated IL-1ra

Absorption pharmacokinetics of pegylated IL-1ra muteins have been characterized following subcutaneous injection of the molecules to rats. Serial blood samples were drawn from the tail vein and assayed for native or pegylated IL-1ra by enzyme-linked immunosorbent assay (ELISA). The resulting subcutaneous plasma IL-1ra concentration vs. time curves are plotted in Figure 11. The subcutaneous

pharmacokinetic data (Table 3) reveal variable systemic availability for the pegylated muteins, related to the site and size of the PEG, and related to subcutaneous injection in non-optimized formulations. Table 3 also reveals a remarkable positive influence of pegylation on the mean residence time for subcutaneously injected IL-1ra. As the size of the PEG is increased, the mean residence time is generally increased. This increase is probably the result of molecule-size-related slower absorption through the lymphatic circulation (longer mean absorption times) as well as to delayed clearance after the pegylated molecule reaches the systemic circulation (plasma). This prolongation is profound and will improve the pharmacokinetic character of subcutaneous IL-1ra in humans.

TABLE 2

	wild type	PEG(8500)	PEG(5000)	C116	C84 PEG(8500) S116	C84 PEG(5000) S116
n	4	1		1	1	1
Vd initial, ml/kg	24	59		38	58	66
Vd steady state, ml/kg	110	160		150	240	290
plasma clearance, ml/min/kg	7.4	3.1		7.7	3.0	5.0
t1/2 initial phase, min	1.7	12		2.5	10	6.5
t1/2 intermediate phase, min	30	60		29	87	82
t1/2 terminal phase, hr	2.0	12		1.9	7.2	5.0
mean residence time, hr	0.25	0.86		0.32	1.4	0.96

EXAMPLE X: PREPARATION OF 30kDa TNF INHIBITOR MUTEINS

Cysteine has been substituted for the native residue at both the amino terminus and carboxyl terminus of the protein as well as all three glycosylation sites (residues 1, 14, 105, 111 and 161 as seen in Figure 2). Mutagenesis was performed on single stranded DNA from the 30kDa TNF inhibitor gene cloned into the bacteriophage M13. This gene is described in detail in U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990. Mutagenesis was done as described by Kunkel et al. (1987) (see Example V). The mutagenized gene was isolated and subcloned into the expression vector pT5T (Eisenberg et al., Nature vol. 343, pg. 341 (1989)) and transformed into the T7 expression system strain E. coli BL21DE3. The 30kDa TNF inhibitor muteins were purified and refolded as described for native 30kDa TNF-inhibitor. See, the U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990. Refolding includes the addition of cysteine to the solution containing the purified protein. The cysteine aids in the refolding and "bonds to" the free cysteine in the mutein.

EXAMPLE XI: PEGYLATION OF 30kDa TNF INHIBITOR MUTEINS

The c105 30kDa TNF Inhibitor mutein was exposed to a 6-fold molar excess of DTT in 50mM HEPES Ph 7.0 for 30 minutes at ambient temperature in order to remove an extra cysteine attached during the refolding process. The protein was then dialyzed against de-gassed 50mM HEPES pH 7.0 for 2 hours to remove the DTT. The c105 30kDa TNF inhibitor was then reacted with a 5 fold molar excess of pegylating reagent 1 (See Example 1A) for 2 hours at ambient temperature in 50mM HEPES pH 7.0. Approximately 60% of the mutein was converted to the pegylated form.

The c105 pegylation reaction mixture was loaded onto a superdex-75 FPLC column (Pharmacia) run at 0.25

ml/min in 50mM Tris pH 7.0, 100mM NaCl. Fractions containing c105-PEG 30kDa TNF-inhibitor were pooled and loaded on a TSK-2000SW HPLC column (Bio-Rad) run at 0.2ml/min in the same buffer. The fractions containing essentially pure c105-PEG 30kDa TNF-inhibitor, as determined by silver stained SDS-PAGE, were pooled and the protein concentration determined by Bio-Rad protein assay. See Figure 9.

The activity was determined using the murine L929 cell TNF cytoxicity assay as described in U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990.

EXAMPLE XII: THE PREPARATION OF BIS-MALEIMIDO PEG

The synthesis of the α-(2-aminoethyl) ω-aminopoly(oxyethylene) derivative of the PEG (hereinafter bisamino PEG) consisted of three steps: 1) sulfonation of the hydroxyl group using tresyl chloride as described by Nilson and Mosback (Nilson et al., Methods in Enzymology vol. 104, pg. 56, Academic Press, Inc., N.Y., N.Y. (1984)), 2) substitution of the tresylated intermediate by phthalimide (Pillai et al., J. Org. Chem. vol. 45, pg. 5364 (1980)), and 3) reduction of the phthalimide intermediate to amine by hydrazine hydrate (Pillai, supra.). Structures of the starting material, intermediates, and products are shown in Appendix 1 to this Example. Optimum conditions permitted a conversion of approximately 80% of the hydroxyl to amine as determined by 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay. The bisamino PEG can be purified from the reaction mixture by ion-exchange chromatography. This is a key step for removing reactive byproducts which can interfere with dimer formation.

The bisamino PEG was acylated using maleic anhydride (Butler et al., Methods in Enzymology vol. 25, pg. 191, Academic Press, Inc., N.Y., N.Y. (1972))

and the resulting intermediate was cyclized to produce α-(2-maleimidoethyl-ω-maleimidopoly(oxyethylene) (Winsch et al., Biol. Chem. Hoppe-Seyler vol. 336, pg. 53 (1985)). This derivative reacts with sulfhydryls via a Michael addition to form a stable thioether.

APPENDIX TO EXAMPLE XII

Starting Material

Generalized formula for polyethylene glycol PEG_x

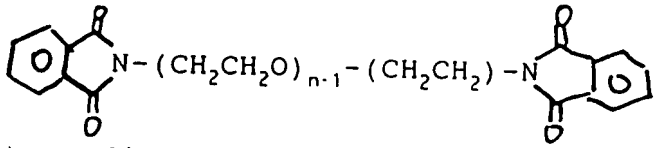
$$HO-(CH_2CH_2O)_n-H$$

where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxyethylene groups.

Intermediate 1

$$F_3-CH_2-SO_2-O-(CH_2CH_2O)_{n-1}-(CH_2CH_2)-O-SO_2-CH_2-F_3$$

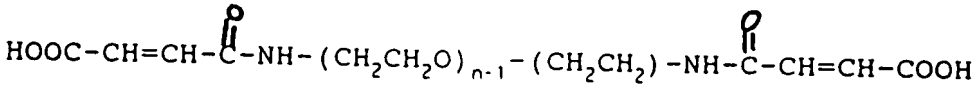
Intermediate 2



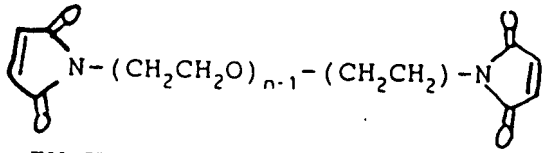
Intermediate 3

$$H_2N-(CH_2CH_2O)_{n-1}-(CH_2CH_2)-NH_2$$

Intermediate 4



O-(2-maleimidoethyl)-O'-methyl-polyethylene glycol



EXAMPLE XIII: IN VIVO RESULTS FOR c105 30kDa TNF INHIBITOR PEG COMPLEXES

The inhibitory effects of four species of

pegylated c105 30kDa TNF inhibitor species were tested in vivo on two different TNF-stimulated physiological actions. One endpoint was the appearance of IL-6 in the plasma of mice that were injected intravenously with human recombinant TNF. The second endpoint was an increase in the migration of neutrophils into the peritoneal cavity after the intraperitoneal administration of human recombinant TNF.

Experiment One. The intravenous administration of c105 30kDa TNF inhibitor ($PEG_{2,000}$, $PEG_{3,500}$, $PEG_{10,000}$) simultaneously with human recombinant TNF inhibits the induction of IL-6 in the plasma of mice.

BALB/c female mice weighing 20 to 23 g were used to measure the induction of plasma IL-6 levels by human recombinant TNF. In a preliminary experiment, the time course was plotted for the appearance IL-6 in the plasma after the intravenous administration via the tail vein of two doses of human recombinant TNF (Figure 12). Peak IL-6 levels occurred at two hours after stimulation with either 10 or 20 ug of human recombinant TNF per mouse. The lower dose was used in subsequent experiments.

The potency of c105 30kDa TNF inhibitor PEG_{2000} dumbbell with that of the unpegylated c105 30kDa TNF inhibitor was compared. Human recombinant TNF was injected intravenously at a dose of 10 ug per mouse either alone or simultaneously with the TNF inhibitors. Four different reactions of inhibitors to TNF were tested (Figure 13). The ratios were calculated based on protein content. Three mice were tested at each dose. Blood was collected at two hours after the intravenous injections. IL-6 levels were measured by ELISA.

Both the c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor PEG_{2000} dumbbell caused nearly complete inhibition of IL-6 levels when administered at

10:1 and 5:1 ratios of inhibitor to TNF. At ratios of 1:1, the c105 30kDa TNF inhibitor PEG_{2000} dumbbell caused 95% reduction of IL-6 levels stimulated by TNF alone, whereas the unpegylated c105 30kDa TNF inhibitor reduced IL-6 by only about 70%. The results of this experiment indicate that in the ratios tested, both the c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor PEG_{2000} dumbbell were good inhibitors of this TNF-stimulated physiological parameter. At a ratio of 1:1, the c105 30kDa TNF inhibitor PEG_{2000} dumbbell caused a greater percentage inhibition than the unpegylated inhibitor.

Two other species of pegylated c105 30kDa TNF inhibitor were tested. The inhibitory effects of c105 30kDa TNF inhibitor $PEG_{3,500}$ dumbbell and c105 30kDa TNF inhibitor $PEG_{10,000}$ dumbbell were tested on plasma IL-6 induction. The inhibitors were administered by intravenous injection simultaneously with human recombinant TNF at ratios of 1:1 (c105 30kDa TNF inhibitor dumbbell: TNF) (Figure 14). Three mice were tested in each of the two inhibitor-treated groups. Ten mice were injected with TNF alone. When administered in ratios of 1:1, no detectable IL-6 was measured in plasma of mice injected with either c105 30kDa TNF inhibitor $PEG_{3,500}$ dumbbell or c105 30kDa TNF inhibitor $PEG_{10,000}$ dumbbell, whereas a significant IL-6 response was elicited in the mice injected with human recombinant TNF alone.

The results of the two experiments show that c105 30kDa TNF inhibitor $PEG_{2,000}$, $PEG_{3,500}$, and $PEG_{10,000}$ dumbbells are good inhibitors of the induction of plasma IL-6 by human recombinant TNF when administered in a low ratio (1:1) relative to the stimulus.

Experiment Two. The subcutaneous administration of c105 30kDa TNF inhibitor ($PEG_{3,500}$, $PEG_{10,000}$ and $PEG_{20,000}$) simultaneously with the

intraperitoneal injection of human recombinant TNF inhibits the migration of neutrophils into the peritoneal cavity.

BALB/c female mice weighing 20 to 23 g were used to measure the migration of neutrophils into the peritoneal cavity after stimulation with human recombinant TNF. The technique used is that of Kim McIntyre et al. (J. Exp. Med. vol. 173, pg. 931 (1991)) and is described in brief herein. Mice are injected with TNF in a volume of 0.1 ml directly into the peritoneal cavity. Four hours later the mice are killed and an immediate post mortem lavage of the peritoneal cavity is performed. Four ml of Hank's Balanced Salt Solution (HBS) (calcium and magnesium free) is injected into the peritoneal cavity. The abdomen is gently massaged. The peritoneal fluid is recovered by aspiration with needle and syringe. The total number of peritoneal cells is counted on a Coulter counter. An aliquot of the cellular suspension is dried on a slide and stained with Diff-Kwik stain. A differential count of the cells is made by direct microscopic examination. One hundred cells are examined and classified as either neutrophils, lymphocytes, or macrophages.

In a preliminary experiment, the compared cellular make-up of the lavage fluid after intraperitoneal administration of either pyrogen-free saline or 7.5 ng human recombinant TNF was compared. TNF caused an increase in the percentage of neutrophils and in the absolute number of neutrophils present in the peritoneal lavage fluid. In saline-treated mice, 9.4×10^4 neutrophils were recovered in the lavage fluid and made up only 2.3% of the total peritoneal cells. In TNF (7.5 ng)-treated mice, the total number of neutrophils was increased to 12.9×10^5 and the percentage of neutrophils was increased to 19.7%.

The potency of unpegylated c105 30kDa TNF

inhibitor with three pegylated species of c105 30kDa TNF inhibitor ($PEG_{3,500}$, $PEG_{10,000}$ and $PEG_{20,000}$ dumbbells) was also compared. Keeping the TNF stimulus constant at 7.5 ng per mouse, the inhibitors were tested at ratios of 100:1, 10:1, and 1:1 (c105 30kDa TNF inhibitor species: TNF). The ratios were calculated based on protein content. The mice were injected subcutaneously with the c105 30kDa TNF inhibitor simultaneous to the intraperitoneal administration of TNF. Six mice were tested in each dose group. Four hours later the peritoneal lavage fluid was collected and analyzed. Values shown in Figure 15 are the percentage neutrophils in the peritoneal lavage fluid. The lowest ratio at which the unpegylated c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor $PEG_{3,500}$ dumbbell significantly inhibited neutrophil migration is 100:1. The c105 30kDa TNF inhibitor $PEG_{10,000}$ and $PEG_{20,000}$ dumbbells significantly inhibited neutrophil migration at a ratio of 10:1.

The results of this experiment show that c105 30kDa TNF inhibitor $PEG_{3,500}$, $PEG_{10,000}$ and $PEG_{20,000}$ dumbbells are good inhibitors of the TNF-stimulated neutrophilic migration into the peritoneal cavity. The c105 30kDa TNF inhibitors $PEG_{10,000}$ and $PEG_{20,000}$ dumbbells were more potent than the unpegylated c105 30kDa TNF inhibitor and the c105 30kDa TNF inhibitor $PEG_{3,500}$.

EXAMPLE XIV: PREPARATION AND BIOACTIVITY OF c105 30kDa TNF INHIBITOR PEG DB

Synthesis

Recombinant c105 30kDa TNF inhibitor 2-3 mg/ml is treated with a 4-fold molar excess of DTT for 2 hrs at ambient temperature. The TNF inhibitor is then dialyzed against de-gassed 50mM HEPES, pH 7.0, for 3 hrs at 4°C. To create the PEG-linked dumbbell, the TNF inhibitor is reacted with different molar ratios of the bis-maleimido PEG in 50 mM HEPES pH 7.0. TNF inhibitor

is reacted with an equimolar ratio of bis-maleimido PEG. The reactions are incubated for 3-12 hrs at ambient temperature. After incubation, the PEG-linked TNF inhibitor dumbbell is purified from un-PEGylated and singly-PEGylated TNF inhibitor using MONO-S FPLC in 50 mM HOAc, pH 4.0, using a 260 mM, 310 mM and 350 mM NaCl step-gradient. The PEG-linked TNF inhibitor dumbbell elutes at the 310 mM NaCl step. Any remaining unPEGylated TNF inhibitor is removed by chromatography on Superdex75.

STEPWISE REAGENT ADDITION:

After DTT treatment and dialysis into 50 mM HEPES pH 7.0, an equimolar amount of bis-maleimido PEG is added, after 1.5 hrs incubation another equimolar amount of bis-maleimide PEG is added. This is incubated for 1.5 hours. This leads to an optimized level of PEG-linked dumbbell formation. Then a 2-fold excess of PEG reagent is added, giving a final PEG-TNF inhibitor ratio of 4:1. This is incubated for 2 hrs and the mixture is dialyzed into 50 mM acetate pH 4.0 for Mono-S chromatography. This yields a mixture which is primarily PEG-linked dimer and singly PEGylated TNF inhibitor. This allow for more efficient purification of PEG-linked dumbbell as there is a greater separation between singly PEGylated TNF inhibitor and dumbbell than dumbbell and unPEGylated TNF inhibitor.

This procedure optimized dumbbell formation, and allowed for more efficient purification.

STEP REACTION:

After DTT treatment and dialysis into 50 mM HEPES pH 7.0 an 8-fold molar excess of bis-maleimido PEG is added. This is incubated for 2 hrs at ambient temperature. This converts essentially all the TNF inhibitor to singly-PEGylated form. The singly-PEGylated TNF inhibitor is separated from PEG reagent

and any remaining unreacted TNF inhibitor using MONO-S HPLC in 50 mM acetate pH 4.0 with a NaCl gradient. The singly-PEGylated material is diafiltered into 50 mM HEPES, pH 7.0, and concentrated to 2-4 mg/ml. DTT treated TNF inhibitor is then added to allow formation of PEG-linked dumbbell. After 2 hrs, the PEG-linked dumbbell is purified using Mono-S HPLC. This method may be used to form a PEG-linked heterodumbbell by adding a second, distinct protein compound.

This procedure optimizes dumbbell formation and can be used for the formation of heterodumbbell compounds. However, this procedure is somewhat labor and time intensive.

Bioactivity of PEG-linked TNF inhibitor Dumbbells

The ability of c105 30kDa TNF inhibitor dumbbells to inhibit the cytotoxicity of TNFα in the murine L929 cell cytotoxicity assay was measured. This has allowed for the determination of an ED_{50} for these molecules. They are as follows:

Wild Type rTNF inhibitor	220 ng/ml
BMH-linked dumbbells	220 ng/ml
1900 MW PEG-dumbbells	4.1 ng/ml
3500 MW PEG-dumbbells	4.8 ng/ml
10,000 MW PEG-dumbbells	4.6 ng/ml
20,000 MW PEG-dumbbells	4.2 ng/ml

The TNF inhibitor dumbbells also have greatly increased activity in inhibiting the cytotoxicity of TNFβ in the L929 bio-assay. The ED_{50} values against TNFβ are as follows:

Wild Type rTNF inhibitor	70 µg/ml
3400 MW PEG-dumbbells	80 ng/ml
20,000 MW PEG-dumbbells	22 ng/ml

EXAMPLE XV: PHARMACOKINETICS OF PEGYLATED 30kDa TNF INHIBITOR

1. Intravenous pharmacokinetics for pegylated 30kDa TNF inhibitor

The pharmacokinetic character of several pegylated 30kDa TNF inhibitor molecules was determined following intravenous administration of the molecules to rats. Native or pegylated TNF inhibitor was injected as an intravenous bolus dose. Serial blood samples were drawn from the tail vein and assayed for non-pegylated or pegylated TNF inhibitor by enzyme-linked immunosorbent assay (ELISA). The resulting intravenous plasma TNF inhibitor concentration vs. time profiles (Figure 16) illustrate that pegylation has a pronounced influence on the disappearance of TNF inhibitor from the plasma after intravenous injection. Statistical moment theory (area under the curve [AUC] and area under the first moment curve [AUMC]) was used to interpret the data of Figure 16. The data indicate that pegylation prolongs the intravenous mean residence time of TNF inhibitor up to fifty-fold in the rat (Table 4). The intravenous mean residence time increases as the size of the attached PEG molecule increases (Table 4). Although not limited by theory, the prolongation of mean residence times may be explained based on conventional pharmacokinetic theory which states that the intravenous mean residence time for a drug is inversely related to the plasma clearance for the drug and directly related to the apparent volume of distribution for the drug. Pharmacokinetic analysis of the disappearance of pegylated TNF inhibitor's from the plasma indicates that the prolongation of half-lives is inversely related to a decreased plasma clearance for the pegylated molecules, compared to non-pegylated TNF inhibitor (Table 4). The decrease in plasma clearance is consistent with an anticipated size-related decrease in glomerular

filtration of the pegylated molecules by the kidneys. Because of the probable qualitative similarity between rats and humans in plasma clearance mechanisms for TNF inhibitor, it is apparent that pegylation will similarly improve the pharmacokinetic properties of TNF inhibitor in humans.

2. Subcutaneous pharmacokinetics for pegylated 30kDa TNF inhibitor

Absorption pharmacokinetics of pegylated TNF inhibitor have been characterized following subcutaneous injection of the molecules to rats. Serial blood samples were drawn from the tail vein and assayed for non-pegylated or pegylated TNF inhibitor concentration vs. time curves and are plotted in Figure 17. The subcutaneous pharmacokinetic data (Table 4) reveal variable systemic availability for the pegylated molecules, related to the size of the PEG, and related to subcutaneous injection in non-optimized formulations. Table 4 also reveals a positive influence of pegylation on the mean residence time for subcutaneously injected TNF inhibitor. As the size of the PEG is increased, the mean residence time is generally increased. While not limited by theory, this increase is likely the result of size-related slower absorption through the lymphatic circulation (longer mean absorption times) as well as delayed clearance once the pegylated molecule reaches the plasma. This prolongation is profound and will improve the pharmacokinetic character of subcutaneous TNF inhibitor in humans.

EXAMPLE XVI. SOLUBILITY OF PEGYLATED PROTEINS

IL-1ra

Results of a solubility study are shown in Figure 18. Solubility curves are shown for three different preparations of IL-1ra, and c84 IL-1ra

PEG_{8500}. The experiments were preformed at 37°C in a microliter plate with all proteins at 160 mg/ml. The plate was sealed with a cover and then read in a plate reader at 405 nm at various time points. An increase in absorbance is an indication of protein precipitation. There is clearly a decrease in the amount of protein falling out of solution for the PEGylated sample relative to native IL-1ra.

30kDa TNF inhibitor

Native 30kDa TNF inhibitor cannot be concentrated to more than 5 mg/ml. Following PEGylation, the solubility was increased at least 5 fold.

EXAMPLE XVII: PREPARATION OF IL-2 INHIBITOR HETERODUMBBELL

A PEG-linked heterodumbbell may be formed by first pegylating IL-2rα in the presence of an excess of bis-maleimido PEG. The singly pegylated IL-2rα may be purified and IL-2rβ added to react with the remaining reactive maleimide group to form the heterodimer.

Potential sites for PEGylation of Il-2rα include both the amino and carboxyl terminal residues, the two N-linked glycosylation sites, as well as the native free cysteine residue in the molecule. Cysteine residue 192 in the soluble extracellular domain of IL-2rα has been identified as being uninvolved in disulfide bonding. (Miedel et al. BBRC, vol. 154, pg. 372 (1988)). This cysteine residue lies in an epitope of an anti-IL-2rα monoclonal antibody that does not affect IL-2 binding to IL-2rα (Lorenzo et al. J. Immunology, vol. 147, pg. 2970 (1991)). This indicates this residue is a likely candidate for PEGylation without affecting the activity of IL-2rα.

For IL-2rβ, the potential sites include both the amino and carboxyl termini, the 4 N-linked glycosylation sites and a region (a.a. #108-118) that

is similar to a region of biological significance in the murine erythropoietin receptor (Yoshimura, Longmore and Lodish, Nature, vol. 348, pg. 647 (1990)). Point mutational analysis of other residues in the receptors may also allow for identification of other sites of PEGylation that yield optimal properties in the heterodumbbell molecule.

EXAMPLE XVIII: PREPARATION OF HETERODUMBBELLS WHICH INHIBIT THE CLASSICAL PATHWAY OF COMPLEMENT SYSTEM

Many proteins which regulate the complement system have been identified and cloned. Some of them are membrane proteins. One of the membrane proteins is called CR1 (complement receptor 1). The soluble form of CR1 has been examined in in vivo models of diseases. The complement inhibitor inhibits post-ischemic myocardial inflammation and necrosis (Weisman et al. Science, vol. 149, pg. 145-151, 1990), reversed passive arthus reaction (Yet et al. J. Immunology, vol. 146, pg. 250-256 (1991)), and allograft rejection (Pruitt et al. J. Surgical Research, vol. 50, pp. 350-355 (1991)).

The soluble CR1 binds to C3b and C4b. It consists of 30 short consensus repeat sequences (SCR). Most of SCR contain one possible glycosylation site and four cysteines. All of the cysteines are likely be to involved in disulfide bonding. SCRs 1-4 are found to be involved in C4b binding. Two separate portions of CR1, SCRs 8-11 and SCRs 15-18, are involved in C3b binding (Klickstein et al. J. Exp. Med., vol. 168, pp. 1699-1717 (1988); Kalli et al. J. Exp. Med. vol. 174, pp. 1451-1460 (1991)). According to this invention, it is possible to produce a heterodumbbell which contains the C4b binding domain and the C3b binding domain of CR1.

The SCRs which contain C4b binding and C3b binding domains of CR1 may be cloned using PCR. These SCRs will be SCRs 1 through 5 (C4b binding) and SCRs 8

through 12 (C3b binding). The genes encoding these SCRs may be cloned in E. coli expression vector. The E. coli expressed-proteins may be refolded and purified. The success of refolding can be analyzed by the capacity to bind polyC3b or polyC4b. In vitro mutagenesis of these genes may be carried out to substitute native amino acid residues to cysteine. These cysteines may then be used to link the PEG molecule. Possible sites for PEGylation will be the glycosylation site or carboxyl terminal residue of SCR 5 and SCR 12. The C4b binding and C3b binding domains which contain an extra cysteine to the carboxyl terminal residue could be constructed and used for linking PEG molecule. The PEG linked heterodumbbell may be produced by the two step process of Example XIV. Purification may be carried out by ion-exchange chromatography.

EXAMPLE XIX: SYNTHESIS OF AN IL-1ra BIS(MALEIMIDE)-PLATELET DERIVED GROWTH FACTOR PEPTIDE PEG HETERODUMBBELL

The platelet derived growth factor (PDGF) peptide YGRPRESGKKRKRKRLKPT is described in Khachigian, L. et al. J. Biol. Chem., vol. 267, pg. 1660-1666 (1991). A terminal C was added to permit coupling to the maleimide.

The heterodumbbell was synthesized in two steps. In the first step, 1.6 nanomoles of IL-1ra suspended in 3 μl of 0.05 M Hepes buffer, pH 7.5, was mixed with 6.4 nanomoles of bis-maleimido PEG_{1900} dissolved in 11 μl of the same buffer. This reaction was carried out for 30 min at 20°C. In the second step, 32 nanomoles of the PDGF peptide dissolved in 4 μl of 0.2 M sodium phosphate buffer, pH 7.0, was added to the products of the first reaction. The reaction was allowed to proceed for 1 hr at 20°C. The reaction was then terminated by the addition of an equal volume of SDS-PAGE sample buffer containing 30 μmoles of 2-

mercaptoethanol.

Samples of the products of the first step of the reaction and the products of the complete two-step reaction, as well as appropriate molecular weight markers, were separated by SDS-PAGE on a 15% polyacrylamide gel which was then stained with Coomassie Blue. The two-step reaction gave an additional band consistent with the predicted size of the heterodumbbell. Approximately 33% of the starting IL-1ra was converted to heterodumbbell by the two-step reaction.

The products of the first step of the reaction can be isolated by cation exchange chromatography on the resin S-Sepharose. The heterodimer may be isolated by cation exchange chromatography due to the abundance of basic amino acids in the peptide.

It is to be understood that the application of the teachings of the present invention to a specific expression system or pegylation reagent will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Thus, it will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the process and products of the present invention. It is intended that the present invention covers these modifications and variations provided they come within the scope of the appended claims and their equivalents.

TABLE 3

Pegylated Interleukin-1 Inhibitor Pharmacokinetics* in rats

type of pegylation =	none	single	single	single	single	single	single	double	dumbbell	dumbbell	dumbbell	dumbbell	dumbbell
PEG location =	[wild	9	84	84	116	116	116	84,116	84	84	84	116	116
PEG size =	type]	8500	5000	8500	5000	8500	12000	8500	**3400	**10000	**20000	3400	**10000
intravenous pharmacokinetics													
Vd initial, mL/kg =	24	51	72	90	52	63	61	50	45	48	58	47	40
Vd steady-state, mL/kg =	110	130	310	290	150	110	130	64	64	68	60	58	44
plasma clearance, mL/min/kg =	7.4	3.3	5.2	4.2	6.8	3.6	2.8	0.30	3.6	0.96	0.37	4.9	1.4
plasma mean residence time (i.v.), hr =	0.25	0.65	1.0	1.2	0.37	0.52	0.76	3.5	0.30	1.2	2.7	0.19	0.52
t1/2 initial phase, min =	1.7	8.8	5.3	11	4.6	11	13						
t1/2 intermediate phase, min =	31	57	38	95	36	36	59	100	7.8	6.2	110	6.4	20
t1/2 terminal phase, hr =	2.8	3.0	2.3	6.9	2.3	3.1	7.0	7.4	0.74	0.87	5.7	1.0	2.6
subcutaneous pharmacokinetics													
systemic availability, % =	68	37	74	99	28	30	30	25	20	7.9	3.3	7.6	4.7
maximum plasma IL-1ra, ng/ml =	1300	530	940	2100	360	540	630	1700	560	630	210	340	230
time of maximum plasma IL-1ra, hr =	0.82	1.8	2.5	4.0	1.5	1.3	1.8	12	3.0	3.1	5.4	0.75	0.51
plasma mean residence time (s.c.), hr =	2.7	8.1	6.4	7.5	6.5	8.5	9.0	21	3.7	6.0	9.7	2.0	7.3
mean absorption time, hr =	2.4	7.4	5.4	6.3	6.1	8.0	8.2	18	3.4	4.8	7.0	1.8	6.7

*each pharmacokinetic parameter is expressed as the mean for two rats, except for C84-PEG(3400) dumbbell s.c. dose, for which n = 1

**pharmacokinetic information is scaled from 1.5 mg/kg to 3 mg/kg dose

TABLE 4

type of pegylation =	none	none	single	single	dumbbell	dumbbell	dumbbell
PEG location =	[wild	C105	C105	C105	C105	C105	C105
PEG size =	type]	0	8500	20000	3500	10000	20000
intravenous pharmacokinetics							
number of animals =		2	2	2	2	2	2
Vd steady-state, mL/kg =		230	240	140	340	93	130
plasma clearance, mL/min/kg =		11.0	1.7	0.17	0.82	0.16	0.11
plasma mean residence time (i.v.), hr =		0.37	2.3	14	6.8	10	19
subcutaneous pharmacokinetics							
number of animals =	2		2	1	1	2	2
systemic availability, % =	99*		25	65	29	39	34
plasma mean residence time (s.c.), hr =	3.5		7.0	20	12	17	30
mean absorption time, hr =	3.1*		4.7	6.0	5.2	7.0	11

* referenced to C105 intravenous pharmacokinetics

CLAIMS

1. A substantially purified compound of the formula R_1-X-R_2 wherein:

R_1 and R_2 are polypeptidic groups; and

X is a non-peptidic polymeric spacer.

2. The substantially purified compound of claim 1 wherein R_1 and R_2 are the same group.

3. The substantially purified compound of claim 1 wherein R_1 and R_2 are different groups.

4. The substantially purified compound of claim 2 wherein R_1 and R_2 are selected from the group consisting of:

IL-1 receptor antagonist, 30kDa TNF inhibitor, 40kDa TNF inhibitor, IL-2 receptor, CR1, PDGF receptor, IL-2, MCSF receptor, EGF receptor, IL-5 receptor, IL-3 receptor, GMCSF receptor, T-cell receptor, HLA-I, HLA-II, NGF receptor, IgG (V_H, V_1), CF40, CD27, IL-6 receptor, Integrins CR3, VLA_4, ICAM, and VCAM, CR2, GMP140 Lec domain, Laminin binding protein, Laminin fragments, Mannose binding protein, exon 6 peptide of PDGF, and proteases.

5. The substantially purified compound of claim 4 wherein R_1 and R_2 are selected from the group consisting of: interleukin-1 receptor antagonist, 30kDa tumor necrosis factor inhibitor, interleukin-2 receptor, and CR1.

6. The substantially purified compound of claim 5 wherein R_1 and R_2 are interleukin-1 receptor antagonist.

7. The substantially purified compound of claim 6

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wherein cysteine residues of said peptidic binding groups R_1 and R_2 are part of said thio-ether bond.

23. The substantially purified compound of claim 1 wherein said polypeptidic binding groups are attached to said non-peptidic polymeric spacer via a cysteine residue.

24. The substantially purified compound of claim 23 wherein said cysteine is not native to the naturally occurring polypeptidic binding group.

25. The substantially purified compound of claim 1 wherein said compound has biological properties distinct from those of R_1 and R_2 alone.

26. The substantially purified compound of claim 2 wherein R_1 and R_2 are receptors.

27. The substantially purified compound of claim 2 wherein R_1 and R_2 are receptor antagonists.

28. The substantially purified compound of claim 2 wherein R_1 and R_2 are binding proteins.

29. The substantially purified compound of claim 2 wherein R_1 and R_2 are selected from the group consisting of receptor antagonists, binding proteins and receptors.

30. The substantially purified compound of claim 1 wherein R_1 and R_2 are biologically active portions of proteins selected from the group consisting of: interleukin-1 receptor antagonist, 30kDA tumor necrosis factor inhibitor, IL-2 receptor, and CR1.

31. A substantially purified com

formula R_1-X-R_2 wherein:

R_1 and R_2 are polypeptidic groups that are proteins or biologically-active portions of proteins selected from the group consisting of interleukin-1 receptor antagonist, 30kDA tumor necrosis factor inhibitor, IL-2 receptors and CR1;

X is a non-peptidic polymer spacer selected from the group consisting of: polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dextran, colonic acids, poly β-amino acids, and carbohydrate polymers, and

R_1 and R_2 are covalently attached to X by thio-ether bonds.

32. A pharmaceutical composition comprised of an effective amount of the substantially purified compound of claim 1 in a pharmacologically acceptable carrier.

33. A method for treating medical indications wherein patients in need thereof are administered the pharmaceutical composition of claim 32.

34. A method for the preparation of substantially purified therapeutically-valuable compounds, comprised of the formula R_1-X-R_2, wherein R_1 and R_2 are cysteine-containing polypeptidic groups and X is a non-peptidic polymeric species comprised of:

reacting a non-peptidic polymeric group having at least two reactive groups capable of forming thio-ether bonds when reacted with cysteine amino acid residues with a cysteine containing polypeptidic group;

isolating and purifying said compound.

35. The method of claim 34 wherein said polypeptidic group is selected from the group consisting of: interleukin-1 receptor antagonist, 30kDa tumor necrosis factor inhibitor, interleukin-2

receptor, and CR1.

36. The method of claim 34 wherein said non-peptidic polymeric group is comprised of a polymeric unit selected from the group consisting of: polyethylene glycol, polypropylene glycol, polyoxethylated glycerol, dextran, colonic acid, poly β-amino acids and carbohydrate polymers.

37. The method of claim 34 wherein said non-peptidic polymeric group is bis-maleimido polyethylene glycol.

38. A method for the preparation of substantially purified therapeutically valuable compounds comprised of the formula R_1-X-R_2, wherein R_1 and R_2 are different, comprised of:

reacting a non-peptidic polymeric group having at least two reactive groups capable of forming thio-ether bonds when reacted with cysteine amino acid residues with a cysteine containing polypeptidic group R_1 to form a complex R_1-X;

reacting complex R_1-X with a cysteine containing polypeptidic group R_2 to form said compound;

isolating and purifying said compound.

39. The method of claim 38 wherein R_1 and R_2 are selected from group consisting of: interleukin-2 receptor antagonist, 30kDa tumor necrosis factor inhibitor, interleukin-2 receptor, CR-1.

40. The method of claim 38 wherein said non-peptidic polymeric group is comprised of a polymeric unit selected from the group consisting of: polyethylene glycol, polypropylene glycol, polyoxethylated glycerol, dextran, colonic acid, poly β-amino acids, and carbohydrate polymers.

41. The method of claim 38 wherein said non-peptidic polymeric group is bismaleimide polyethylene glycol.

42. A substantially purified compound prepared by the method of claim 34.

43. A substantially purified compound prepared by the method of claim 38.

44. Native interleukin-1 receptor antagonist wherein cysteine residue 116 is covalently attached to a non-peptidic polymer.

45. The native interleukin-1 receptor antagonist of claim 44 wherein said polymer is monomethoxy polyethylene glycol.

46. The native interleukin-1 receptor antagonist of claim 44 wherein two native interleukin-1 receptor antagonists are bound to said non-peptidic polymer.

47. A mutein of interleukin-1 receptor antagonist wherein native interleukin-1 receptor antagonist is modified to contain at least one non-native cysteine residue.

48. The mutein of claim 47 wherein said non-native cysteines are found at amino acid residue sites selected from the group consisting of 0, 84, 6, 8, 9 and 141.

49. The mutein of claim 47 wherein the cysteine at position 116 of native interleukin-1 receptor antagonist is replaced with another amino acid residue.

50. The mutein of claim 47 wherein at least one

cysteine residue is covalently attached to non-peptidic polymer.

51. The mutein of claim 49 wherein at least one of said non-native cysteine residues is covalently attached to a non-peptidic polymer.

52. The mutein of claim 47 wherein two muteins are bound to a non-peptidic polymer.

53. The mutein of claim 48 wherein two muteins are bound to a non-peptidic polymer.

54. A mutein of 30kDa tumor necrosis factor inhibitor wherein native 30kDa tumor necrosis factor inhibitor is modified to contain at least one non-native cysteine residue.

55. The mutein of claim 54 wherein said non-native cysteine is found at amino acid residue sites selected from the group consisting of 1, 14, 105, 111 and 165.

56. The mutein of claim 54 wherein at least one of said non-native cysteine residues is covalently attached to a non-peptidic polymer.

57. The mutein of claim 54 wherein two muteins are bound to a non-peptidic polymer.

58. A method for the preparation of therapeutically- valuable polypeptides having an increased apparent molecular weight comprised of:

altering the gene coding for said polypeptide by site directed mutagenesis to create a gene coding for a mutein of said polypeptide containing at least one

non-native cysteine residue;

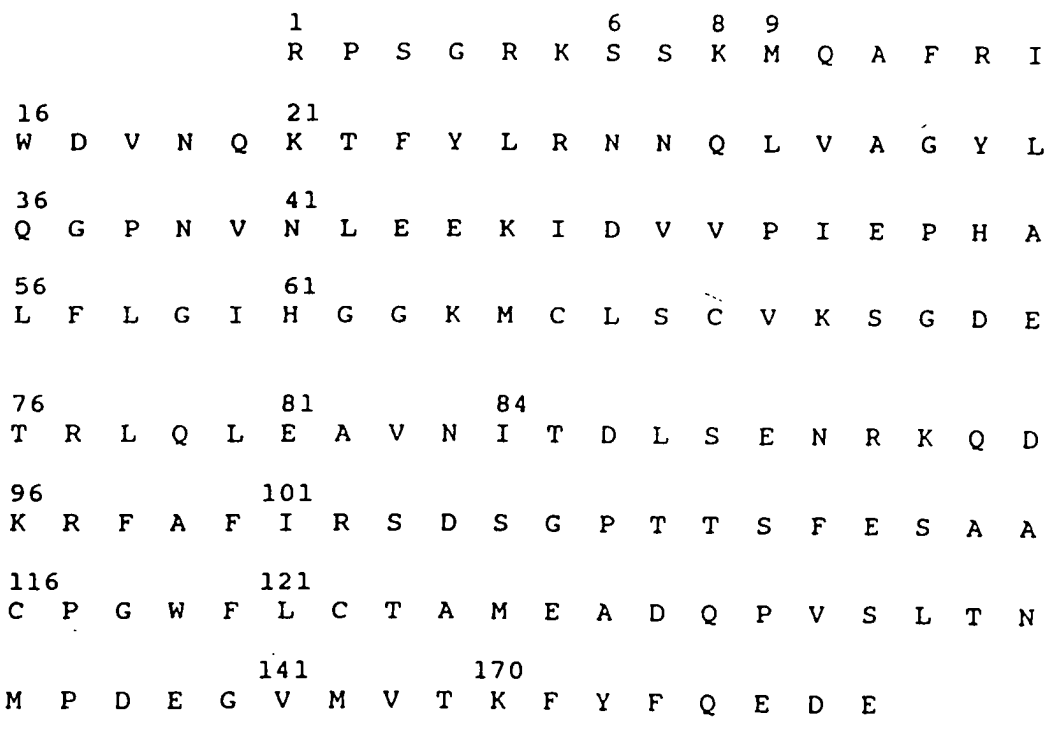
expressing said altered gene in a bacterial expression system;

purifying said expressed mutein;

refolding said mutein in the presence of a sulfhydryl-containing compound;

reducing said refolded mutein with a mild reducing agent to free said non-native cysteines; and

reacting said mutein with a non-peptidic polymer group containing an activating group that is sulfhydryl specific.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16 21
W D V N Q K T F Y L R N N Q L V A G Y L
36 41
Q G P N V N L E E K I D V V P I E P H A
56 61
L F L G I H G G K M C L S C V K S G D E
76 81 84
T R L Q L E A V N I T D L S E N R K Q D
96 101
K R F A F I R S D S G P T T S F E S A A
116 121
C P G W F L C T A M E A D Q P V S L T N
141 170
M P D E G V M V T K F Y F Q E D E

FIG. 1

```
                                                                  20
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
                                                                  40
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
                                                                  60
Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu
                                                                  80
Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
                                                                 100
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu
                                                                 120
Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
                                                                 140
Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
                                                                 160
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu
161
Asn
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FIG.2

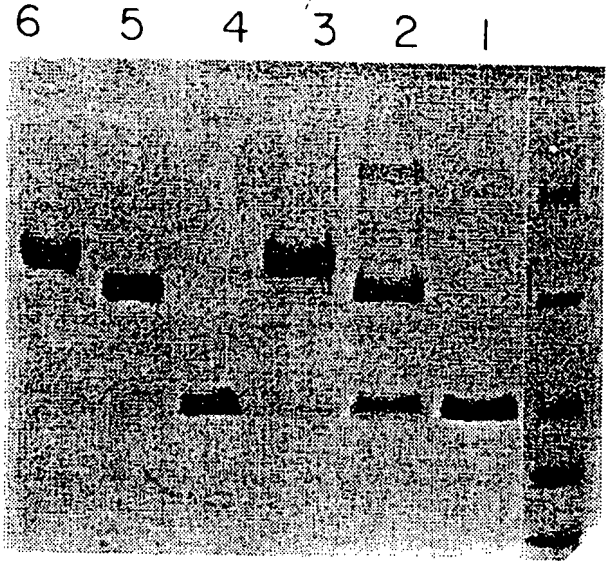


FIG. 3

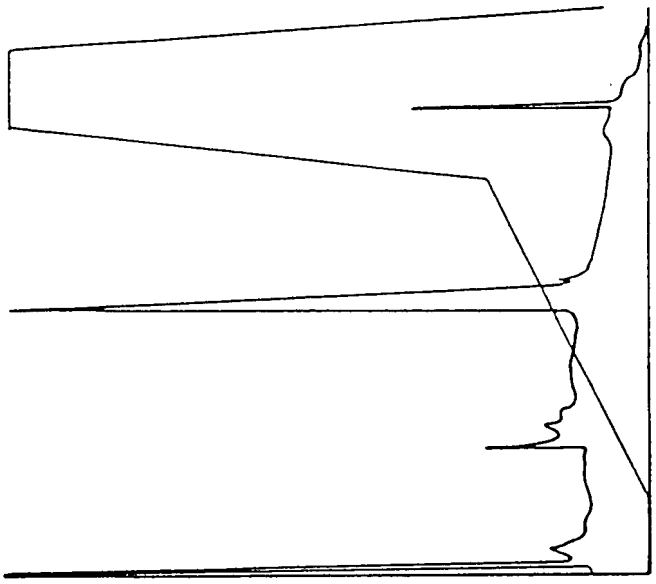


FIG.4B

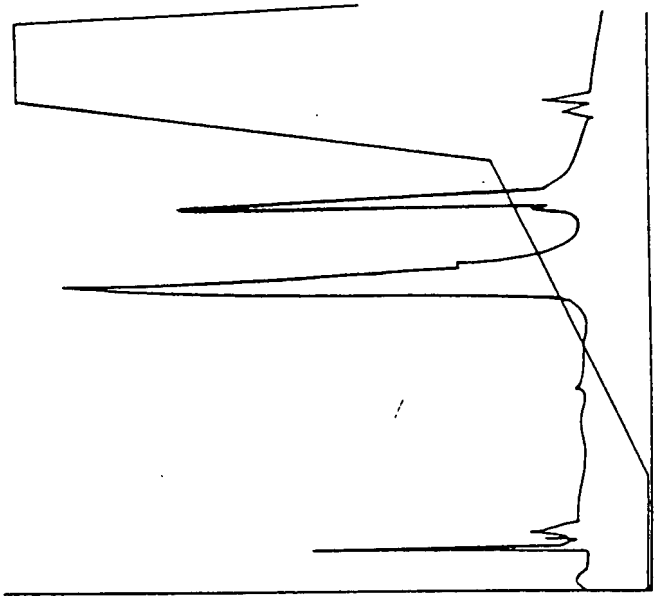


FIG.4A

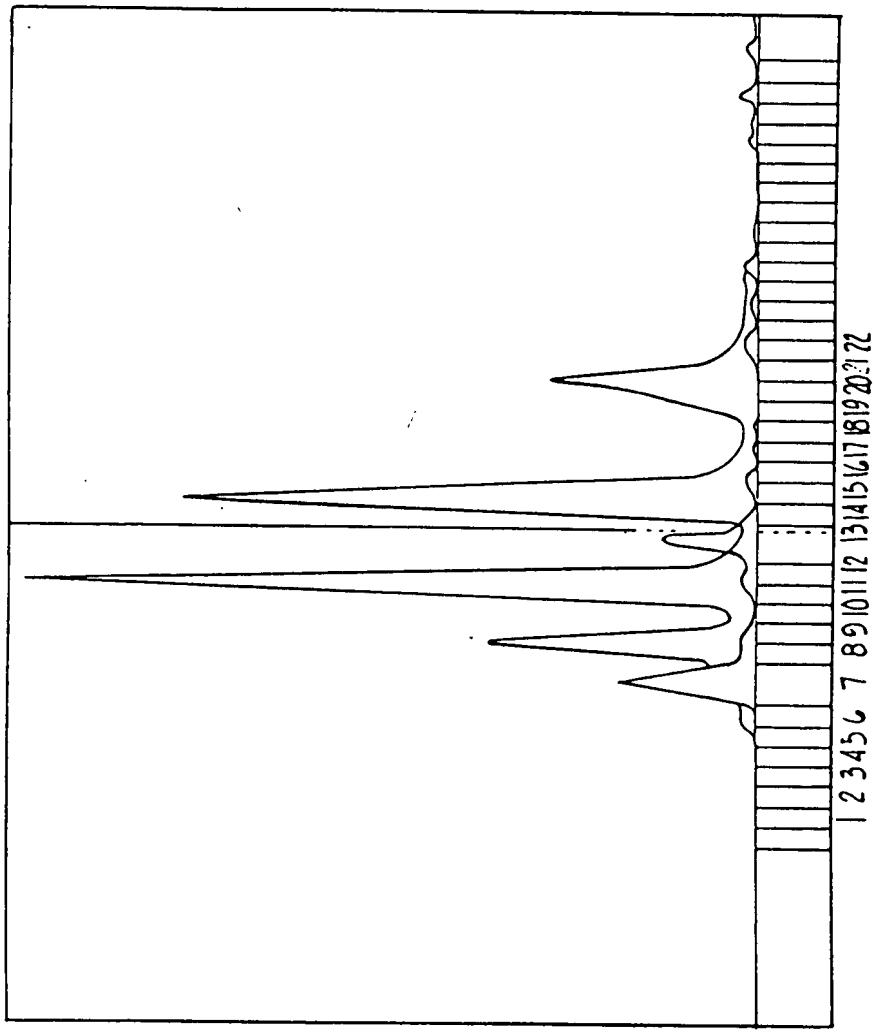


FIG. 5

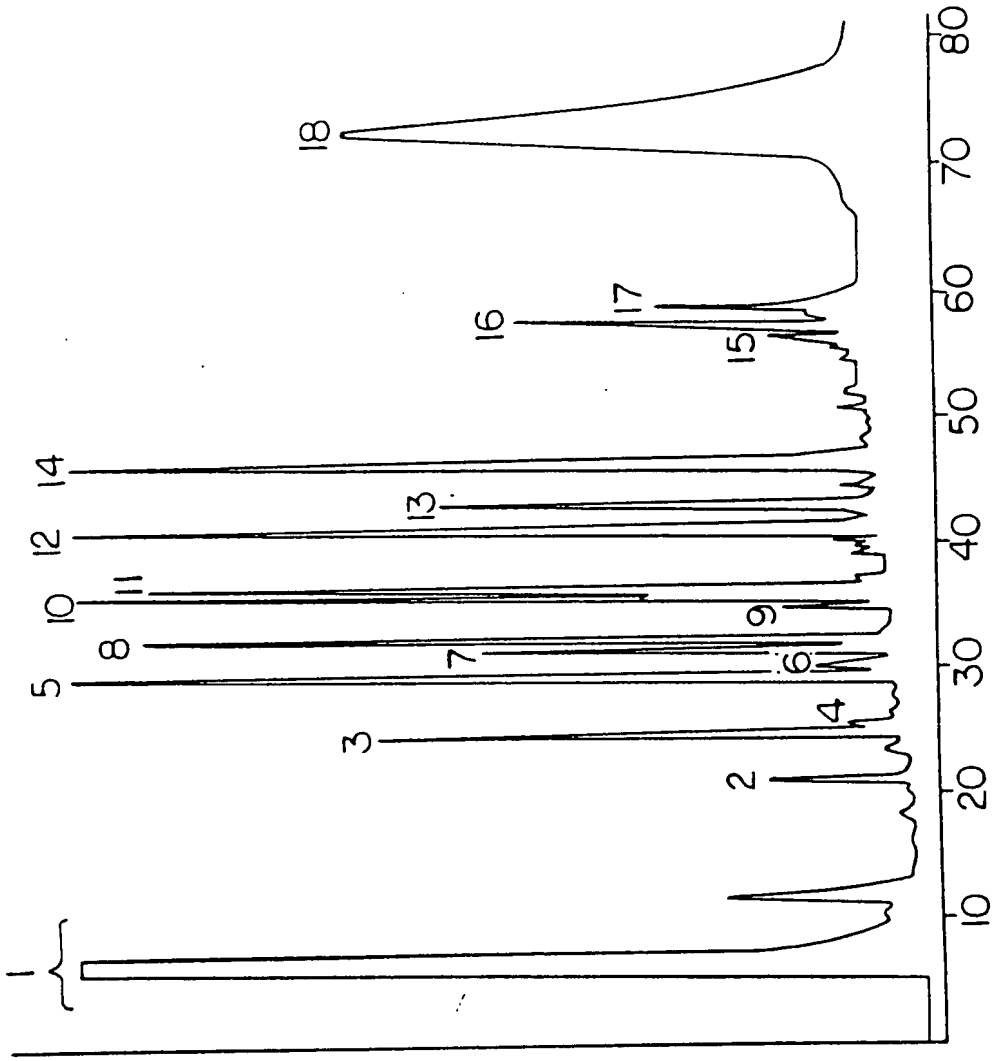


FIG.6

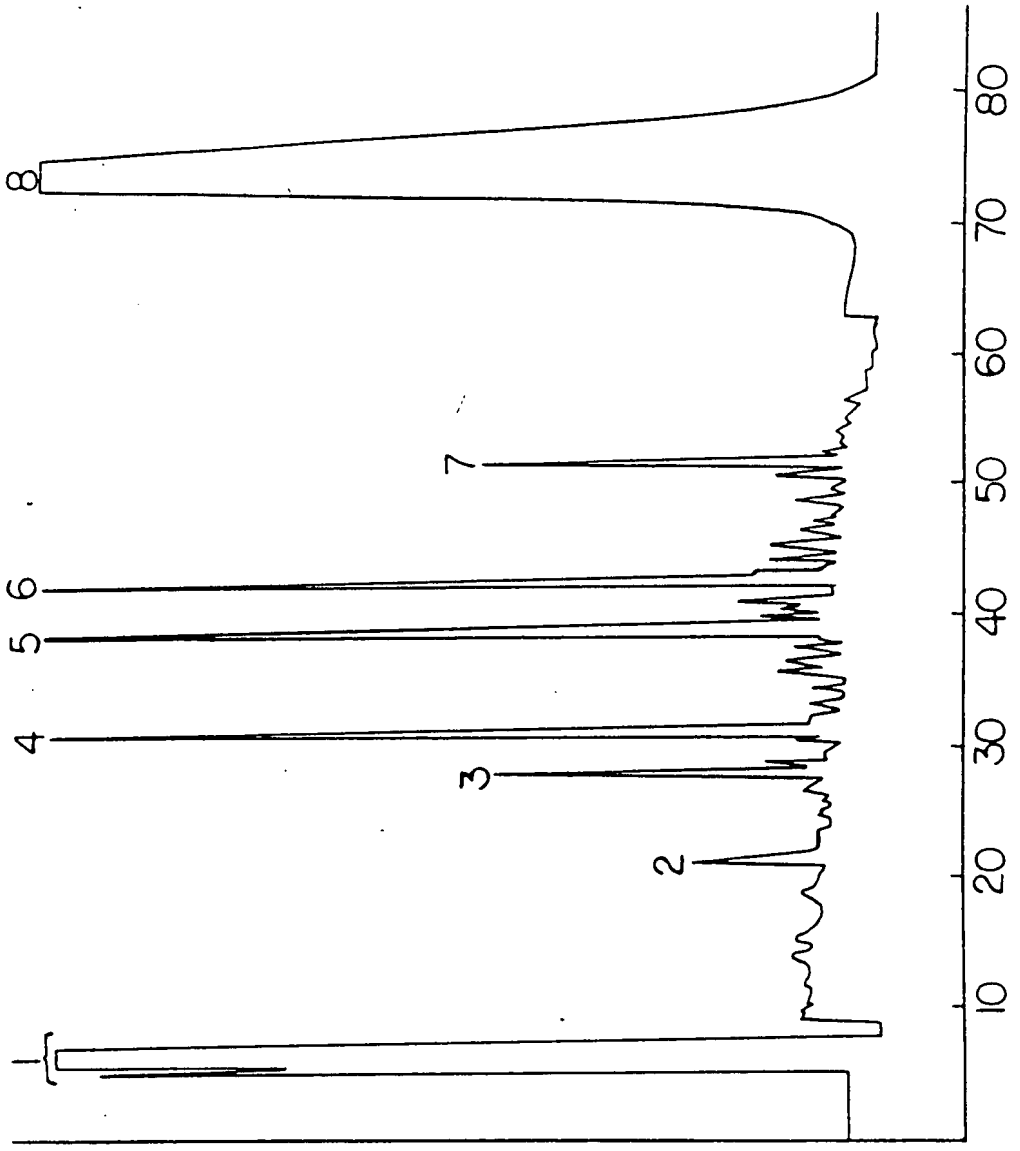


FIG. 7

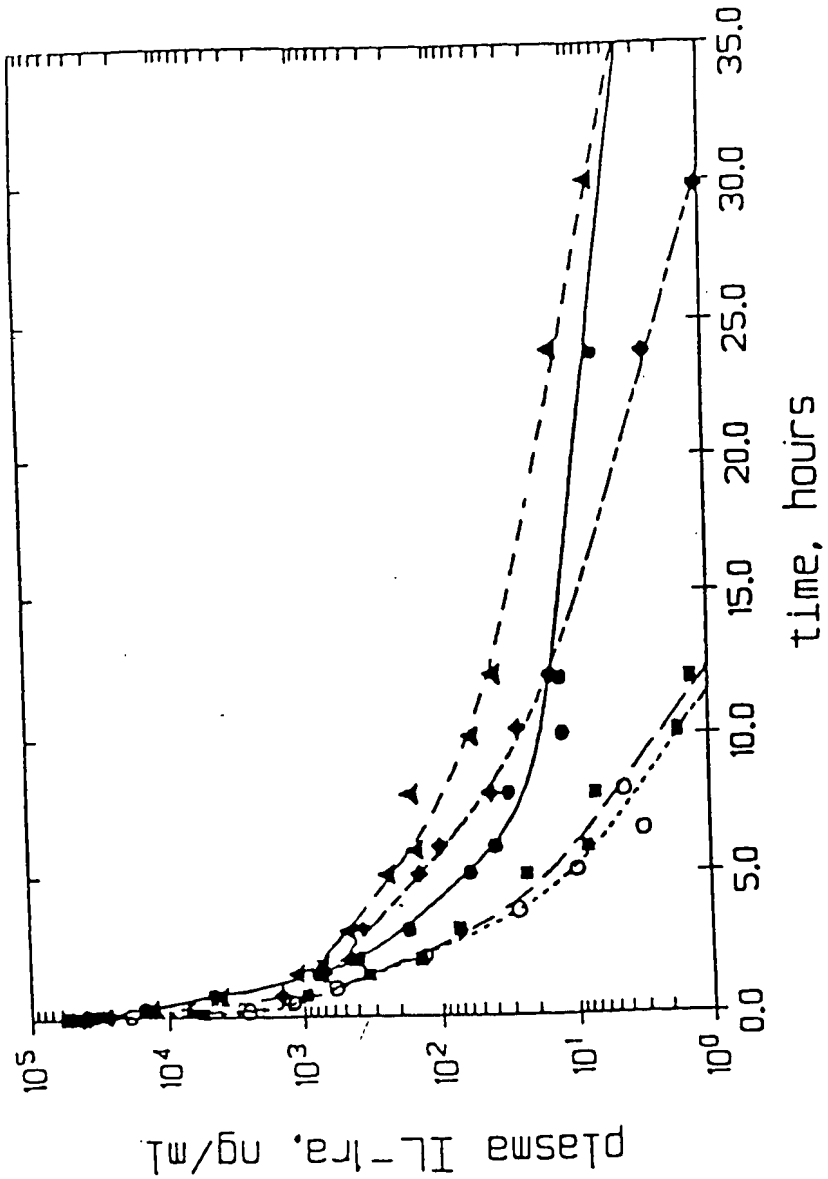

plasma IL-1ra, ng/mL
10^5
10^4
10^3
10^2
10^1
10^0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
time, hours

FIG.8

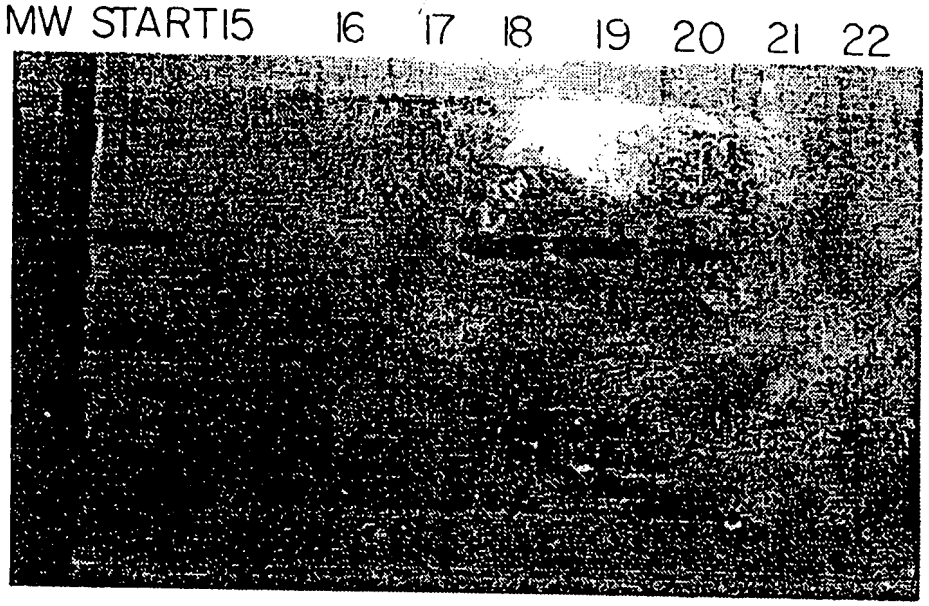


FIG. 9

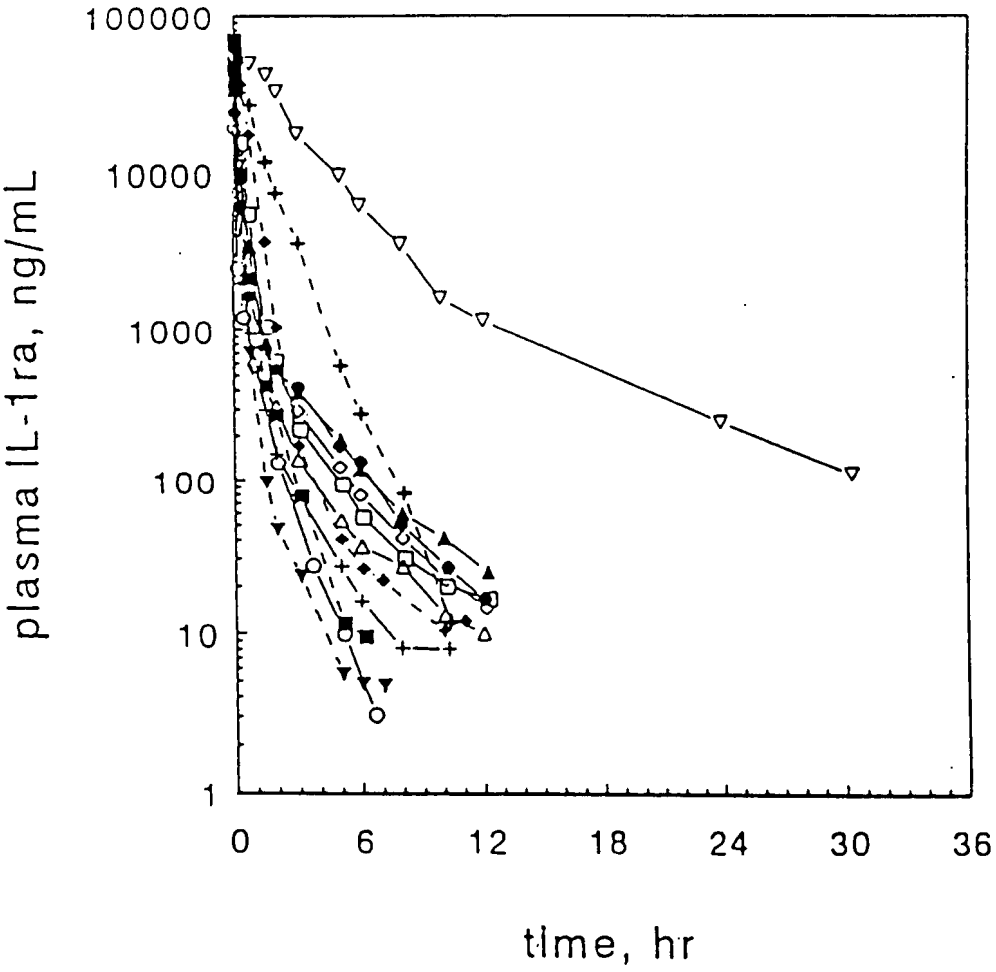

100000
10000
1000
100
10
1
plasma IL-1ra, ng/mL
0
6
12
18
24
30
36
time, hr

FIG.10

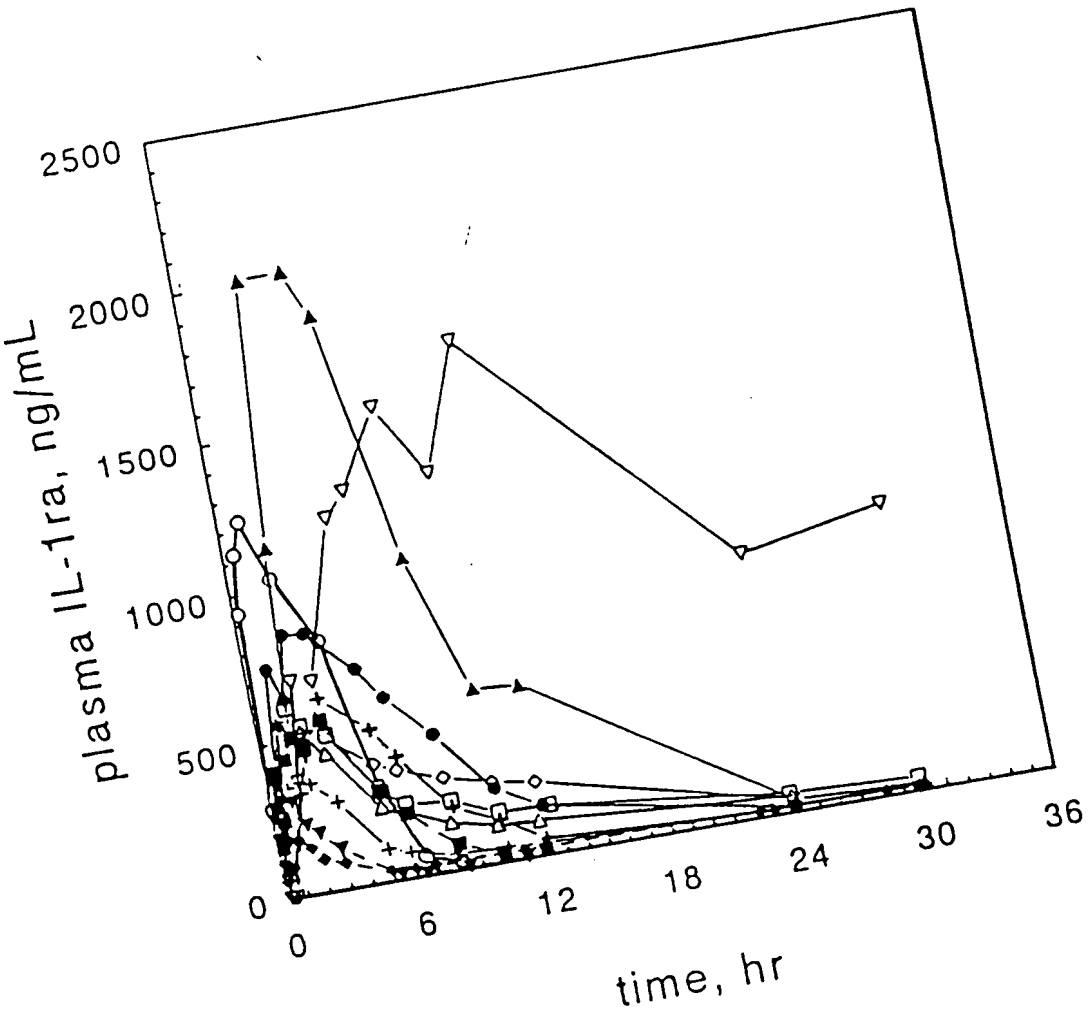

2500
2000
1500
1000
500
0
plasma IL-1ra, ng/mL
0
6
12
18
24
30
36
time, hr

FIG.11

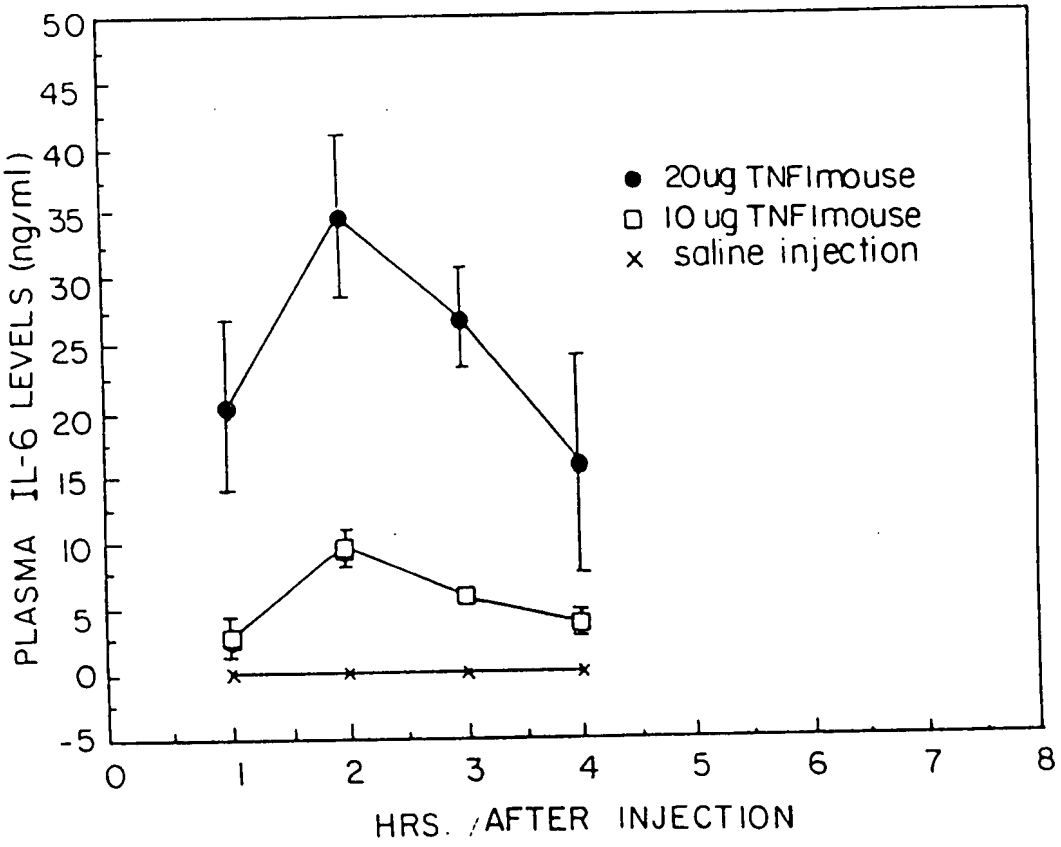

PLASMA IL-6 LEVELS (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
-5
0
1
2
3
4
5
6
7
8
HRS. AFTER INJECTION
20ug TNF/mouse
10 ug TNF/mouse
saline injection

FIG. 12

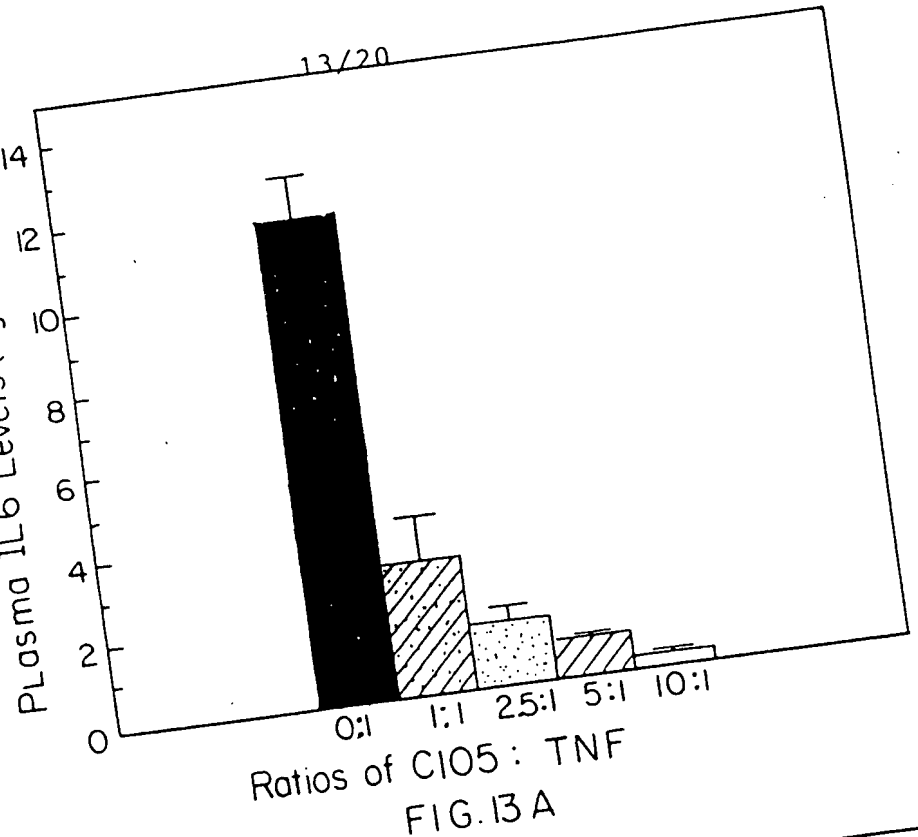


FIG. 13 A

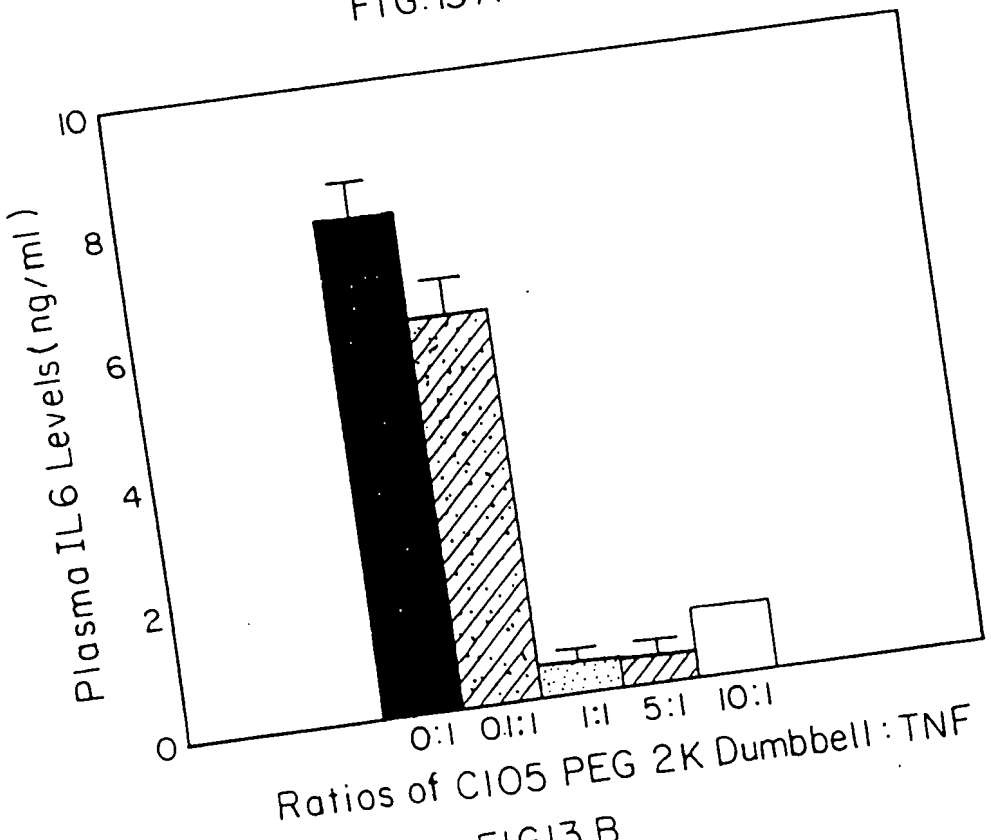


FIG.13 B

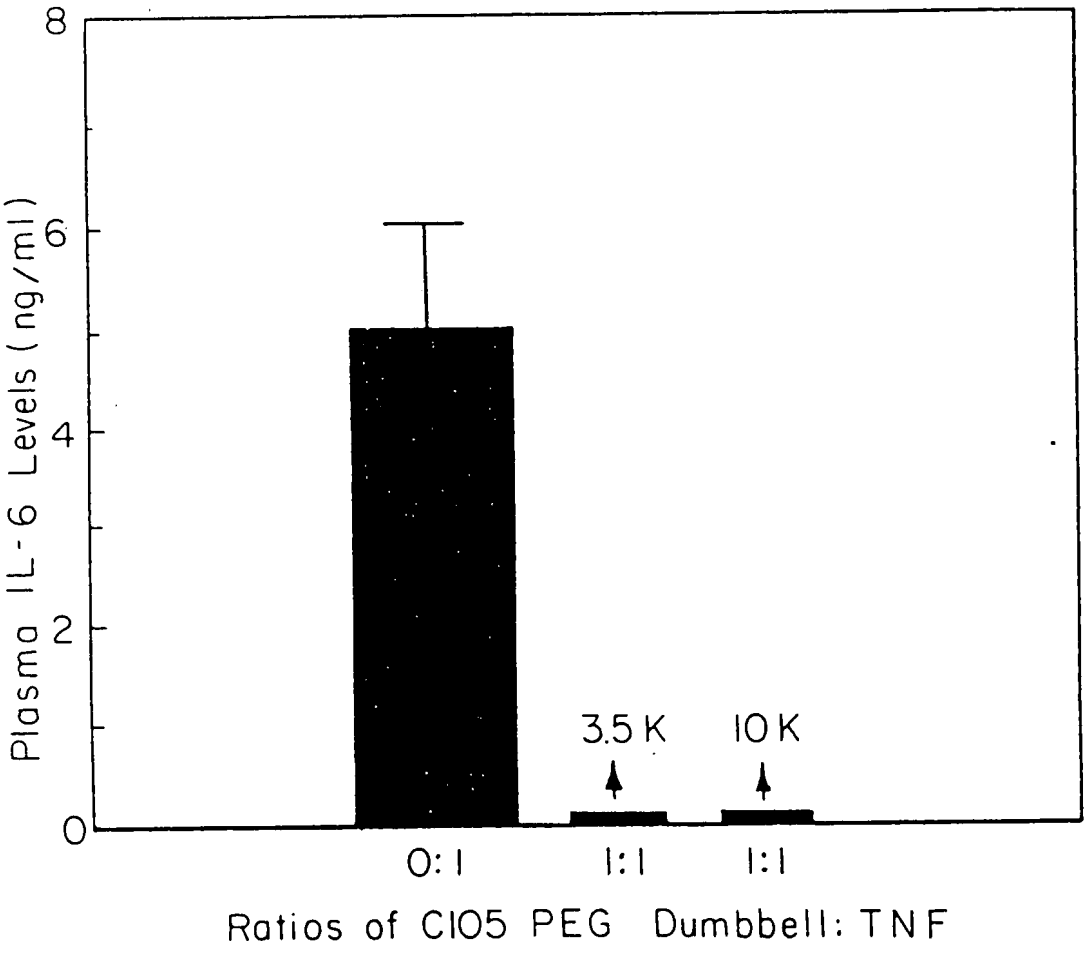

8
6
4
2
0
Plasma IL-6 Levels (ng/ml)
3.5 K
10 K
0:1
1:1
1:1
Ratios of C105 PEG Dumbbell: TNF

FIG.14

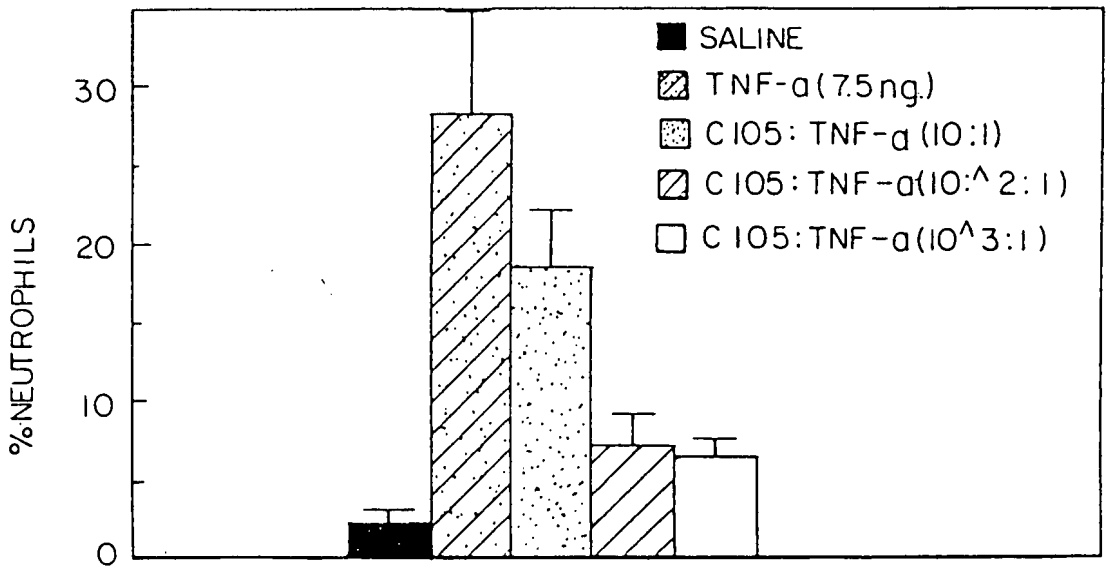


FIG 15A

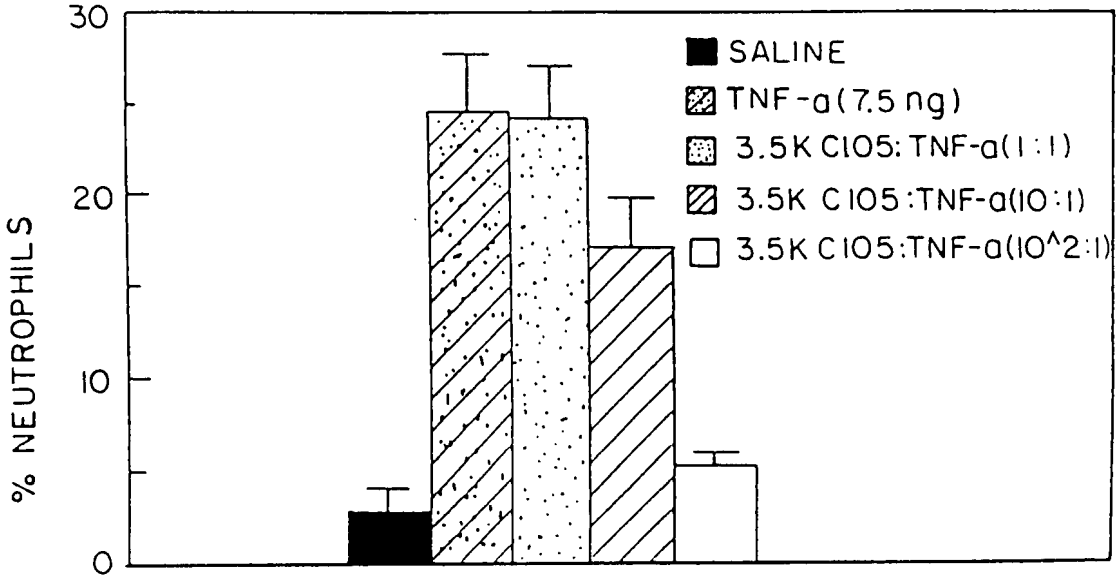


FIG.15B

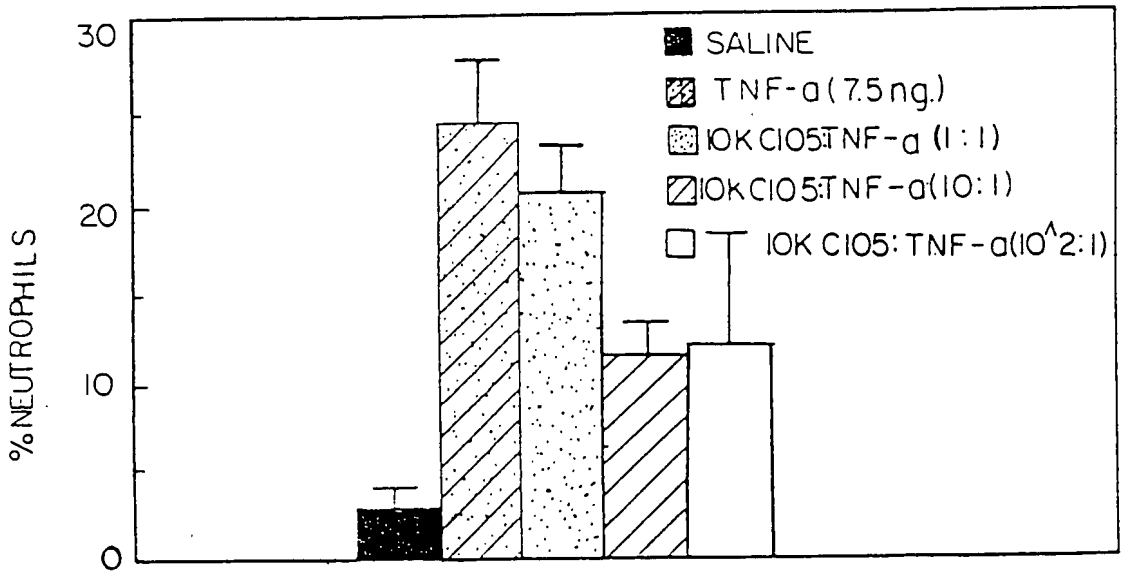


FIG 15C

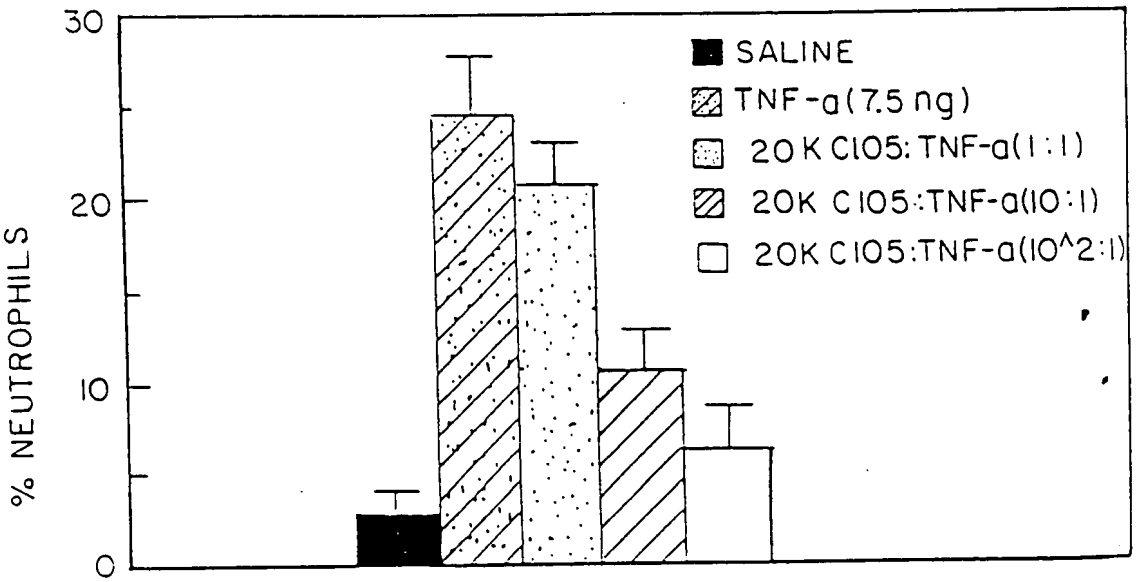


FIG.15D

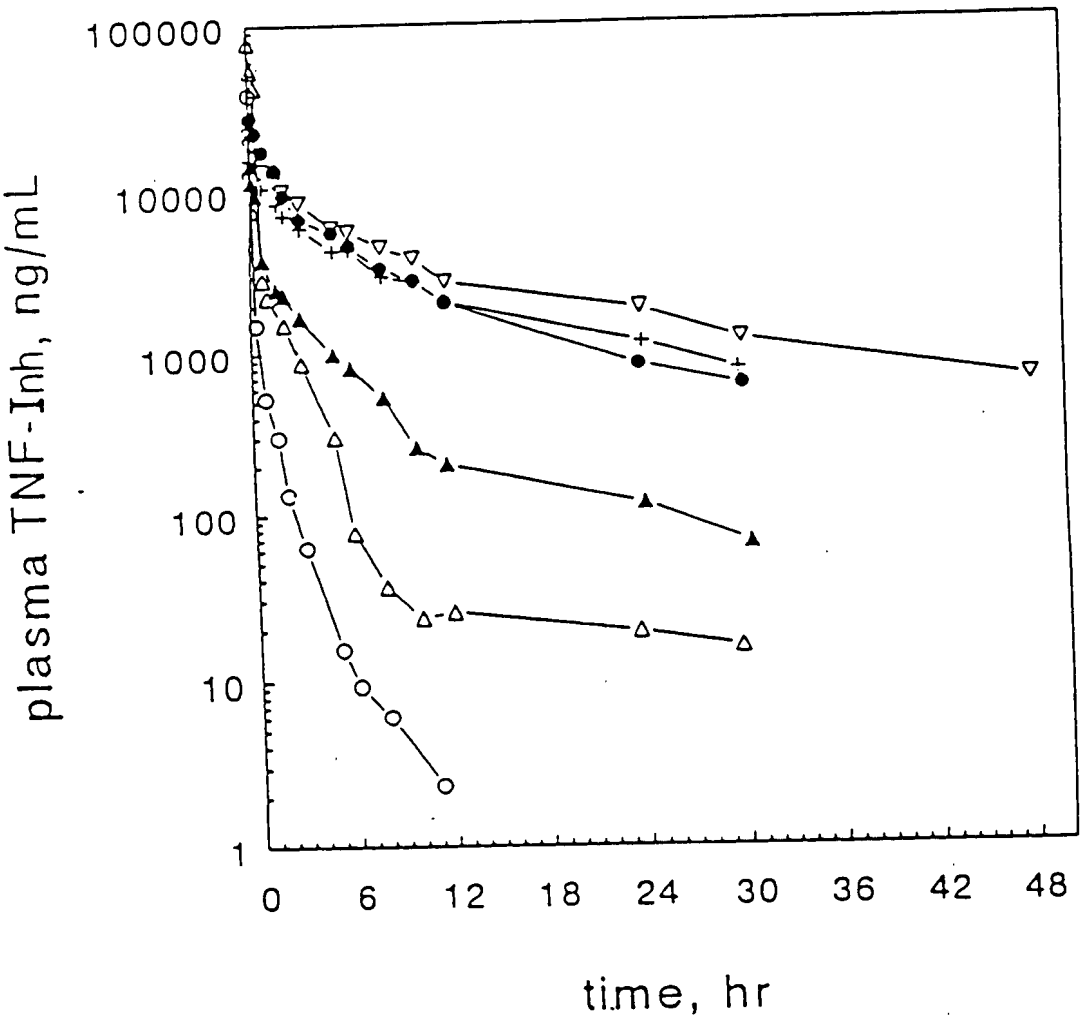

100000
10000
1000
100
10
1
plasma TNF-Inh, ng/mL
0
6
12
18
24
30
36
42
48
time, hr

FIG. 16

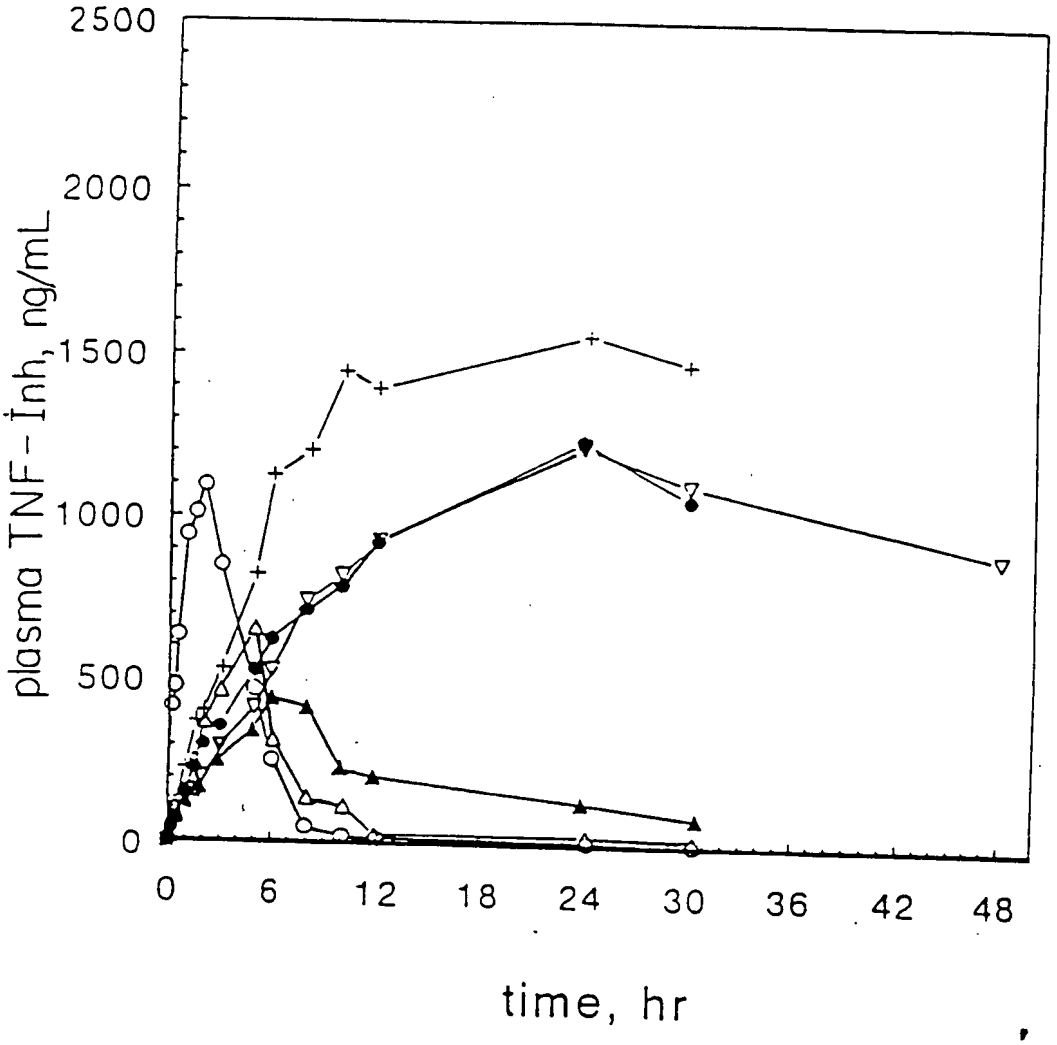

2500
2000
1500
1000
500
0
plasma TNF-Inh, ng/mL
0
6
12
18
24
30
36
42
48
time, hr

FIG. 17

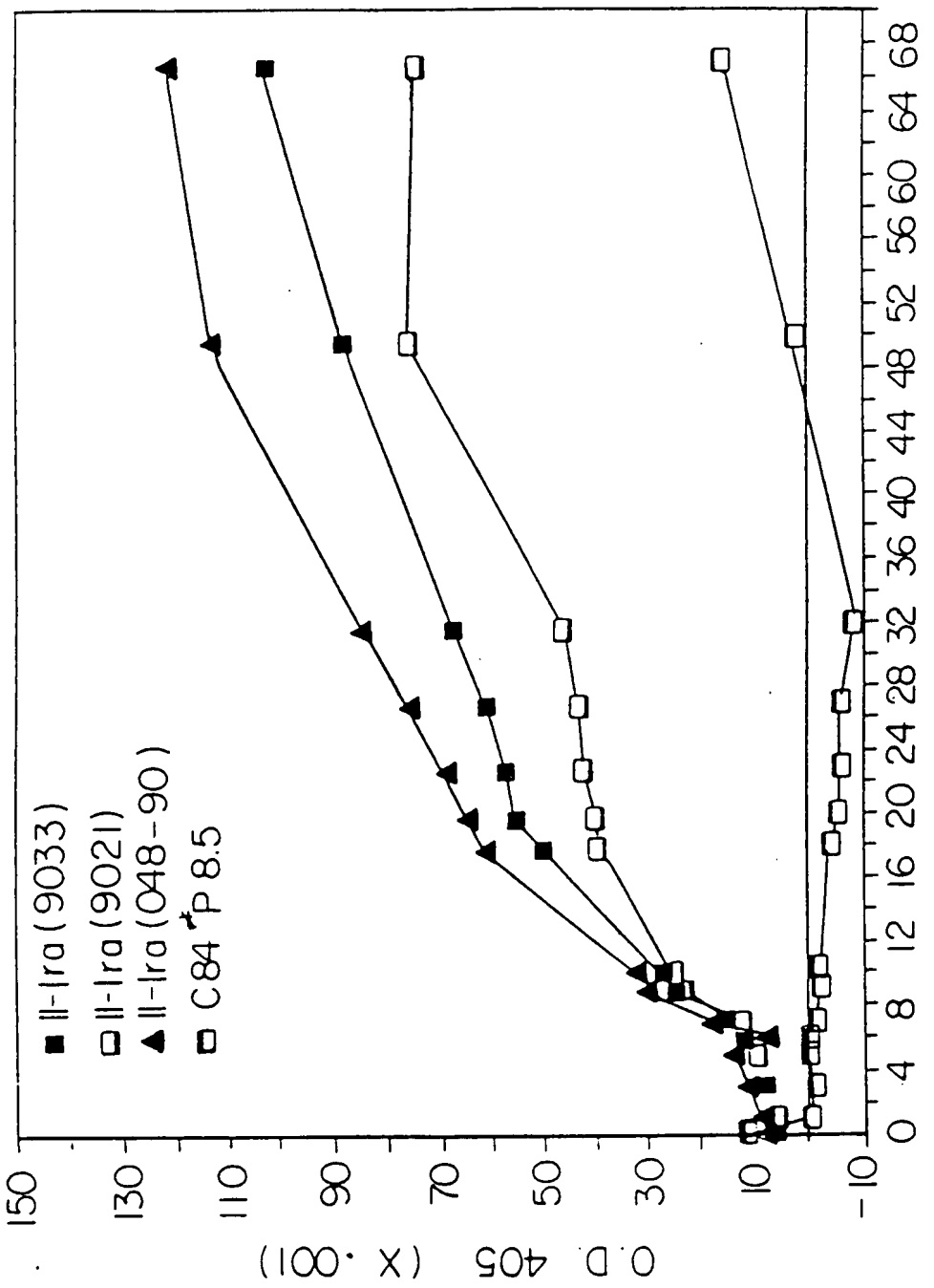

O.D 405 (X .001)
150
130
110
90
70
50
30
10
-10
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68
TIME (HRS.)
II-1ra (9033)
II-1ra (9021)
II-1ra (048-90)
C84 P 8.5

FIG.18

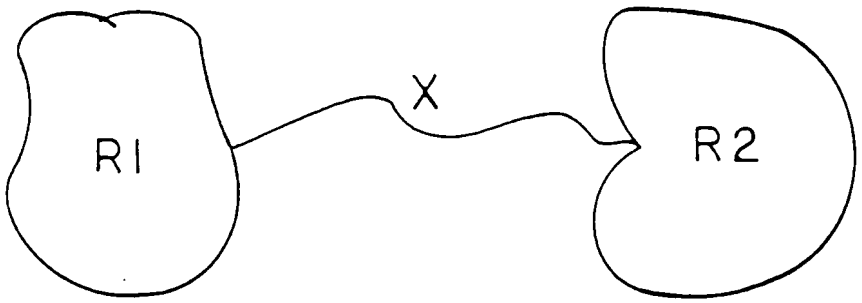


FIG.19

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-57, drawn to pegylated proteins, method of making same and use thereof, Class 514, subclass 12, Class 435 subclasses 69.7 and 240.2, and Class 530, subclasses 410, 395 and 399.
II. Claim 58, drawn to a method of making muteins, Class 435, subclasses 172.1 and 71.2, and Class 530, subclass 402.

The claims of groups I and II are drawn to distinct methods and have a separate status in the art as shown by their different classification. Group I is drawn to the chemical linkage of proteins, whereas Group II is drawn to site-directed mutagenesis for the production of muteins. PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.

PEGYLATION REAGENTS AND COMPOUNDS FORMED THEREWITH

Cross Reference to Related Applications

This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/151,481, filed November 12, 1993, and a continuation-in-part of co-pending U.S. Patent Application Serial No. 07/850,675, filed March 13, 1992, which is a continuation-in-part of abandoned U.S. Patent Application Serial No. 07/669,862, filed March 15, 1991, which is a continuation-in-part of abandoned U.S. Patent Application Serial No. 07/555,274, filed July 19, 1990 and a continuation-in-part of U.S. Patent No. 5,075,222, issued December 24, 1991, all of which are incorporated herein by reference.

Field of the Invention

This invention relates to active derivatives of polyethylene glycol and related hydrophilic polymers and to methods for their synthesis for use in modifying the characteristics of surfaces and molecules. The invention also relates to polypeptides that have been covalently bonded to such active derivatives and methods for making the same.

Background of the Invention

Polyethylene glycol ("PEG") has been studied for use in pharmaceuticals, on artificial implants, and other applications where biocompatibility is of importance. Various derivatives of PEG have been proposed that have an active moiety for permitting PEG to be attached to pharmaceuticals and implants and to molecules and surfaces generally. For example, PEG derivatives have been proposed for coupling PEG to surfaces to control wetting, static

buildup, and attachment of other types of molecules to the surface, including proteins or protein residues.

PEG derivatives have also been proposed for affinity partitioning, for example, of enzymes from a cellular mass. In affinity partitioning, the PEG derivative includes a functional group for reversible coupling to an enzyme that is contained within a cellular mass. The PEG and enzyme conjugate is separated from the cellular mass and then the enzyme is separated from the PEG derivative, if desired.

In still further examples, coupling of PEG derivatives ("PEGylation") is desirable to overcome obstacles encountered in the clinical use of biologically active molecules. Published PCT Publication No. WO 92/16221 states, for example, that many potentially therapeutic proteins have been found to have a short half life in the blood serum. For the most part, proteins are cleared from the serum through the kidneys. The systematic introduction of relatively large quantities of proteins, particularly those foreign to the human system, can give rise to immunogenic reactions that, among other problems, may lead to rapid removal of the protein from the body through formation of immune complexes. For other proteins, solubility and aggregation problems have also hindered the optimal formulation of the protein.

PEGylation decreases the rate of clearance from the bloodstream by increasing the apparent molecular weight of the molecule. Up to a certain size, the rate of glomerular filtration of proteins is inversely proportional to the size of the protein. The ability of PEGylation to decrease clearance, therefore, is generally not a function of how many PEG groups are attached to the protein, but the overall molecular weight of the altered protein. Decreased clearance can lead to increased efficiency over the non-PEGylated material. See, for example, Conforti et al., Pharm. Research Commun. vol. 19, pg. 287 (1987) and Katre et al., Proc. Natl. Acad. Sci. U.S.A. vol. 84, pg. 1487 (1987).

In addition, PEGylation can decrease protein aggregation (Suzuki et al., Biochem. Biophys. Acta vol. 788, pg. 248 (1984)), alter protein immunogenicity (Abuchowski et al., J. Biol. Chem. vol. 252 pg. 3582 (1977)), and increase protein solubility as described, for example, in PCT Publication No. WO 92/16221.

PEGylation of proteins illustrates some of the problems that have been encountered in attaching PEG to surfaces and molecules. The vast majority of PEGylating reagents react with free primary amino groups of the polypeptide. Most of these free amines are the epsilon amino group of lysine amino acid residues. Typical proteins possess a large number of lysines. Consequently, random attachment of multiple PEG molecules often occurs leading to loss of protein activity.

In addition, if the PEGylated protein is intended for therapeutic use, the multiple species mixture that results from the use of non-specific PEGylation leads to difficulties in the preparation of a product with reproducible and characterizable properties. This non-specific PEGylation makes it difficult to evaluate therapeutics and to establish efficacy and dosing information. The site selective PEGylation of such proteins could lead to reproducibly-modified materials that gain the desirable attributes of PEGylation without the loss of activity.

The need to reproducibly create complexes of two or more linked bioactive molecules or compounds also exists. In certain cases, the administration of multimeric complexes that contain more than one biologically active polypeptide or drug leads to synergistic benefits. For example, a complex containing two or more identical binding polypeptides may have substantially increased affinity for the ligand or active site to which it binds relative to the monomeric polypeptide. Alternatively, a complex comprised of (1) a bioactive protein that

exerts its effect at a particular site in the body and (2) a molecule that can direct the complex to that specific site may be particularly beneficial.

A need also exists for hydrolytically-stable activated polymers which form linkages which are also hydrolytically stable. Otherwise, in certain cases, the reactive group can be rendered inactive before the desired reaction takes place or the conjugate formed after reaction has a short half life in aqueous media, such as blood or plasma.

For example, Zalipsky U.S. Patent No. 5,122,614 describes that PEG molecules activated with an oxycarbonyl-N-dicarboximide functional group that can be attached under aqueous, basic conditions by a urethane linkage to the amine group of a polypeptide. Activated PEG-N-succinimide carbonate is said to form stable, hydrolysis-resistant urethane linkages with amine groups. The amine group is shown to more reactive at basic pHs of about 8.0 to 9.5, and reactivity falls off sharply at lower pHs. Hydrolysis of the uncoupled PEG derivative, however, also increases sharply at pHs of 8.0 to 9.5. Zalipsky avoids the problem of an increase in the rate of reaction of the uncoupled PEG derivative with water by using an excess of PEG derivative to bind to the protein. By using an excess of PEG derivative, sufficient reactive amino sites are bound to PEG to modify the protein before the PEG derivative becomes hydrolyzed and unreactive.

Zalipsky's method is adequate for nonspecific attachment of the lysine fraction of a protein to a PEG derivative at one active site on the PEG. If the rate of hydrolysis of the PEG derivative is substantial, however, then it can be problematic to provide attachment at more than one active site on the PEG molecule, since a simple excess does not slow the rate of hydrolysis.

For example, a linear PEG with active sites at each end will attach to protein at one end but the reactive site at the other end can react with water to form a relatively nonreactive

hydroxyl moiety instead of a PEG linking two protein groups. A similar problem arises if it is desired to couple a molecule to a surface by a PEG linking agent because the PEG is first attached to the surface or couples to the molecule, and the opposite end of the PEG derivative must remain active for a subsequent reaction. If hydrolysis is a problem, then the opposite end typically becomes inactivated.

Zalipsky U.S. Patent No. 5,122,614 also describes several other PEG derivatives from prior patents. PEG-succinoyl-N-hydroxysuccinimide ester is said to form ester linkages that have limited stability in aqueous media. PEG-cyanuric chloride is said to be toxic and is non-specific for reaction with particular functional groups on a protein which can lead to protein inactivation. PEG-phenylcarbonate is said to produce toxic hydrophobic phenol residues that have an affinity for proteins. PEG activated with carbonyldiimidizole is said to be too slow in reacting with protein functional groups, requiring long reaction times to obtain sufficient modification of the protein.

Still other PEG derivatives have been proposed for attachment to functional groups other than the epsilon amino group of lysine. Maleimide, for example, is specific for cysteine sulfhydryl but the maleimide functionality is subject to hydrolysis.

Accordingly, a need exists for reagents and methods for reproducibly creating complexes whose parts are linked by nonantigenic, highly soluble, biologically inert molecules. The present invention satisfies the need for such complexes and provides related advantages. The present invention also satisfies the need for hydrolytically stable reagents that form hydrolytically stable conjugates.

Summary of the Invention

The present invention relates to biologically-active conjugates containing a biologically-active molecule having a reactive thiol moiety and a non-peptidic polymer having an active sulfone moiety which forms a link with the reactive thiol moiety. The biologically-active molecule can be a synthetic, a naturally occurring, or a modified naturally occurring molecule. A molecule possessing the desired biological activity can be modified to contain a reactive thiol moiety.

Particularly useful biologically active molecules include the tumor necrosis factor ("TNF') inhibitors, Interleukin-1 receptor antagonists ("IL-1ra's"), CR1, exon six peptide of PDGF, and the Interleukin-2 ("IL-2") inhibitors and receptors ("IL-2r").

The polymer of the present invention contains at least one active sulfone moiety and has the formula P-SO_2-C-C*- , where P is polymer and C* is a reactive site for linkage with thiol moieties. The link between the thiol and activated sulfone is at C* and can be represented by the formula P-SO_2-C-C*S-R, where R is the biologically-active molecule. Useful activated sulfone moieties include, for example, vinyl sulfone and chloroethyl sulfone.

Various polymers can be activated for use in all embodiments of the present invention including water soluble polymers such as polyethylene glycol ("PEG") and related hydrophilic polymers.

The present invention also provides methods of using sulfone-activated polymers to make the biologically-active conjugates discussed above. The method includes the steps of:

(a) reacting the biologically-active molecule having a reactive thiol moiety with a non-peptidic polymer having an active sulfone moiety to form a conjugate; and

(b) isolating the conjugate.

Pharmaceutical compositions containing the conjugates are also within the scope of the invention.

The present invention further relates to sulfone-activated polymers useful for coupling to a variety of molecules, compounds, and surfaces. The activated sulfone moiety is the same as discussed above. Particularly useful activated polymers include bifunctional PEG derivatives activated with a sulfone moiety at one site on the PEG molecule and an NHS-ester or a maleimide functionality at another site.

Further included in the present invention are substantially purified biologically-active compounds having the formula R_1-X-R_2 , called a "dumbbell" where at least one of R_1 or R_2 is a biologically-active molecule which retains its biological activity when part of the compound. The biologically-active molecule has a reactive thiol moiety which forms a link with a Michael acceptor group on a non-peptidic polymer. Biologically-active molecules suitable for use in the present invention include those mentioned above. Useful Michael acceptor groups include, for example, vinyl sulfone and maleimide. Polymers which can be activated with Michael acceptor functional groups include the water soluble polymers mentioned above.

R_1 and R_2 can be the same or different moieties. When the R groups are the same, the compound is a homodumbbell; when the R groups are different, the compound is a heterodumbbell. Particularly useful homodumbbells include, for example, PEG-linked TNF inhibitors and PEG-linked IL-1ra's. Useful heterodumbbells include, for example, those formed from IL-2r-α and IL-2r-β, heterodumbbells which inhibit the classical pathway of the complement system, and heterodumbbells formed from IL-1ra and exon 6 of PDGF.

Methods of making the dumbbell compounds are within the scope of the invention. The methods of making a dumbbell, R_1-X-R_2, include the steps of:

(a) reacting X with R_1 and R_2 to form R_1-X-R_2; and

(b) purifying R_1-X-R_2.

Step (a) in the above methods of making dumbbells can further include the following steps:

protecting one reactive group of X to form a protected group on X;

reacting X having a protected group with R_1 to form R_1-X;

deprotecting the protected group on X; and

reacting R_1-X with R_2 to form R_1-X-R_2.

Alternatively or in addition, step (a) can further include the following steps:

reacting an excess of X with R_1 to form R_1-X; and

reacting R_1-X with R_2 to form R_1-X-R_2.

Pharmaceutical compositions containing the substantially purified compounds R_1-X-R_2 are also within the scope of the invention.

Detailed Description

The present invention provides biologically-active conjugates containing (1) a biologically-active molecule having a reactive thiol moiety, and (2) a non-peptidic polymer having an active sulfone moiety which forms a linkage with the thiol moiety of the biologically-active molecule.

A "conjugate" means a complex that is formed by joining a biologically-active molecule, having an active thiol moiety, to a non-peptidic polymer, having an active sulfone moiety, via a linkage between the thiol and sulfone. As stated above, the conjugates of the present invention are biologically active.

"Biologically active" means capable of exerting a biological effect, in vitro or in vivo. A biologically active molecule includes, but is not limited to, any compound that can induce a biological effect on interaction with a natural biological molecule or on a biological system

such as a cell or organism. Ways of demonstrating biological activity include in-vitro bioassays, many of which are well known in the art. For example, one can measure the biological activity of tumor necrosis factor ("TNF") inhibitors by determining if the inhibitors bind to TNF or if the inhibitors block TNF-mediated lysis of certain cells. The latter bioassay is set forth in published European Patent Application No. 90113673.9, which is specifically incorporated herein by reference.

Biologically-active molecules include, but are not limited to, pharmaceuticals, vitamins, nutrients, nucleic acids, amino acids, polypeptides, enzyme co-factors, steroids, carbohydrates, organic species such as heparin, metal containing agents, receptor agonists, receptor antagonists, binding proteins, receptors or portions of receptors, extracellular matrix proteins, cell surface molecules, antigens, haptens, targeting groups, and chelating agents. All references to receptors include all forms of the receptor whenever more than a single form exists.

"Polypeptides" and "proteins" are used herein synonymously and mean any compound that is substantially proteinaceous in nature. However, a polypeptidic group may contain some non-peptidic elements. For example, glycosylated polypeptides or synthetically modified proteins are included within the definition. "Targeting groups" can direct a compound to a location in a biological system. Binding proteins and receptors can be described by their affinity for a certain ligand.

Many polypeptides useful in the present invention are set forth in published PCT Publication No. WO 92/16221, specifically incorporated herein by reference. These proteins are well known in the art. Particularly useful polypeptides are the TNF binding proteins, also called TNF inhibitors. A "TNF binding protein" is defined herein to mean a protein that binds TNF.

One TNF binding protein ("TNFbp") is the extracellular portion of the p55 TNF receptor or the TNF receptor I. In vivo, the extracellular portion of the receptor is shed and circulates in the bloodstream as a 30kDa glycosylated protein which binds to TNF. This binding protein is also referred to TNFbp-I or the 30kDa TNFbp. The purification and amino acid and nucleic acid sequences of this TNF binding protein are set forth in published European Patent Application No. 90 113 673.9, which is incorporated herein by reference.

This published reference also teaches the recombinant production of glycosylated and deglycosylated forms of this TNF inhibitor. Although the actual molecular weight of the deglycosylated form of this inhibitor is approximately 18kDa, the term "30kDa TNF inhibitor" includes the glycosylated and deglycosylated forms.

As used herein, the terms "naturally-occurring," "native," and "wild-type" are synonymous.

European Patent Application No. 90 113 673.9, incorporated herein by reference, also sets forth the purification and amino acid and nucleic acid sequences of another TNF inhibitor, called the 40kDa TNF inhibitor. Also called TNFbp-II, this inhibitor, in its naturally-occurring form, is the glycosylated extracellular portion of the p75 or p85 TNF receptor. European Patent Application No. 90 112 673.9 also teaches the recombinant production of the glycosylated and deglycosylated forms of this "40kDa" inhibitor. The nucleic and amino acid sequences of the native 40kDa TNF inhibitor are set forth in this published reference. Although the molecular weight of the deglycosylated form is not 40kDa, both the glycosylated and deglycosylated forms of this TNFbp are referred to as "40kDa TNF inhibitor."

European Patent Application No. 90 112 673.9, incorporated herein by reference, further teaches the recombinant production of two TNF inhibitors which are portions of the

full length "40kDa" binding protein. These two truncates are called the "Δ51" and "Δ53" TNF inhibitors. The amino acid and nucleic acid sequences of the Δ51 and Δ53 inhibitors are set forth in this published reference.

Other particularly useful polypeptides include the interleukin-1 receptor antagonists ("IL-1ra's"), as described in U.S. Patent No. 5,075,222, incorporated herein by reference, insulin-like growth factor binding proteins ("IGFbps"), CTLA4, and exon six of platelet derived growth factor ("PDGF"), glial derived neurotrophic factor ("GDNF"), ciliary neurotrophic factor ("CNTF"), interleukin-4 receptor ("IL-4r), and inhibitors, and interleukin-1 receptor ("IL-2r"). The nucleic acid encoding the naturally occurring IL-1ra and a method for expressing the protein in E. Coli. are set forth in United States Patent No. 5, 075, 222 of Hannum et al..

Characteristics of the IL-2 receptors and CR1, the nucleic acids encoding them, and methods for their production are discussed in published PCT Publication No. WO 92/16221, specifically incorporated herein by reference.

The biologically-active molecules linked to polymers in the conjugates of the present invention have a reactive thiol moiety prior to forming the linkage. A "reactive thiol moiety" means a -SH group capable of reacting with the activated polymers as described herein.

An example of a reactive thiol is the -SH of the amino acid cysteine. Many proteins do not have free cysteines (cysteines not involved in disulfide bonding) or any other reactive thiol group. In addition, the cysteine thiol may not be appropriate for linkage to the polymer because the thiol is necessary for biological activity. In addition, proteins must be folded into a certain conformation for activity. In the active conformation, a cysteine can be inaccessible for reaction with sulfone because it is buried in the interior of the protein. Moreover, even an accessible cysteine thiol which is not necessary for activity can be an inappropriate site to

form a linkage to the polymer. Amino acids not essential for activity are termed "nonessential." Nonessential cysteines can be inappropriate conjugation sites because the cysteine's position relative to the active site results in the polypeptide becoming inactive after conjugation to polymer. Like proteins, many other biologically-active molecules have reactive thiols which, for reasons similar to those recited above, are not suitable for conjugation to the polymer or contain no reactive thiol groups.

Accordingly, the present invention contemplates the introduction of reactive thiol groups into a biologically-active molecule when necessary or desirable. Thiol groups can also be introduced into an inactive molecule to form a biologically-active molecule as long as the thiol-sulfone link does not destroy the desired activity.

Reactive thiol groups can be introduced by chemical means well known in the art. Chemical modification can be used with polypeptides or non-peptidic molecules and includes the introduction of thiol alone or as part of a larger group, for example a cysteine residue, into the molecule. An example of chemically introducing thiol is set forth in Jue, R. et al., Biochemistry, 17, pp. 5399-5406 (1978). One can also generate a free cysteine in a polypeptide by chemically reducing cystine with, for example, DTT.

Polypeptides which are modified to contain an amino acid residue in a position where one was not present in the native protein before modification is called a "mutein." To create cysteine muteins, a nonessential amino acid can be substituted with a cysteine or a cysteine residue can be added to the polypeptide. Potential sites for introduction of a non-native cysteine include glycosylation sites and the N or C terminus of the polypeptide. The mutation of lysine to cysteine is also appropriate because lysine residues are often found on the surface of a protein in its active conformation. In addition, one skilled in the art can use any

information known about the binding or active site of the polypeptide in the selection of possible mutation sites.

One skilled in the art can also use well known recombinant DNA techniques to create cysteine muteins. One can alter the nucleic acid encoding the native polypeptide to encode the mutein by standard site directed mutagenesis. Examples of standard mutagenesis techniques are set forth in Kunkel, T.A., Proc. Nat. Acad. Sci., Vol. 82, pp. 488-492 (1985) and Kunkel, T.A. et al., Methods Enzymol., Vol. 154, pp. 367-382 (1987), both of which are incorporated herein by reference. Alternatively, one can chemically synthesize the nucleic acid encoding the mutein by techniques well known in the art. DNA synthesizing machines can be used and are available, for example, from Applied Biosystems (Foster City, CA). The nucleic acid encoding the desired mutein can be expressed in a variety of expression systems, including animal, insect, and bacterial systems.

When the mutein is recombinantly produced in a bacterial expression system, the following steps are performed:

1) The nucleic acid encoding the desired mutein is created by site directed mutagenesis of the nucleic acid encoding the native polypeptide;

2) The nucleic acid encoding the desired mutein is expressed in a bacterial expression system;

3) The mutein is isolated from the bacteria and purified;

4) If not folded properly, the mutein is refolded in the presence of cysteine or another sulphydryl containing compound;

5) The refolded mutein is isolated and purified;

6) The purified and refolded target mutein is treated with a mild reducing agent;

7) The reaction mixture is dialyzed in the absence of oxygen.

As discussed below, the mutein can be isolated from the reaction mixture prior to conjugation with polymer but need not be. A reducing agent particularly useful in step 6 is dithiothreitol ("DTT") or Tris-(carboxyethylphosphine) ("TCEP"). TCEP is useful because it does not have to be removed before conjugation with a thiol-specific PEG reagent. See Burns, J.A. et al., J. Org. Chem., Vol.56, No. 8, pp. 2648-2650 (1991).

After creation of the desired mutein, one skilled in the art can bioassay the mutein and compare activity of the mutein relative to the native polypeptide. As more fully discussed below, even if the relative activity of the mutein is diminished, the conjugate formed from the mutein can be particularly useful. For example, the conjugate can have increased solubility, reduced antigenicity or immunogenicity, or reduced clearance time in a biological system relative to the unconjugated molecule. Such improvements in the pharmacokinetic performance of the biologically-active molecule can increase the molecule's value in various therapeutic applications. Increased solubility can also improve the value of the molecule for in-vitro diagnostic applications.

Table 1 lists muteins of IL-1ra that have been produced. The preparation and purification of IL-1ra muteins are set forth in published PCT Patent Publication No. WO 92/16221, specifically incorporated herein by reference. The residue numbering is based upon the sequence set forth in that published application with "0" denoting addition of an amino acid at the N-terminus; "c" referring to cysteine and "s" referring to serine. For example, "c0s116" means a cysteine was inserted at the N terminus and a serine was inserted at position 116. Native IL-1ra has free cysteine residues at positions 66, 69, 116 and 122.

TABLE 1. MUTEINS OF IL-1ra

c0s116	c0
c84s116	c6
c8s116	c8
c9s116	c9
c141s116	c141

Table 2 shows muteins of the 30kDa TNF inhibitor which have also been prepared. The native 30kDa TNF inhibitor, unlike IL-1ra, does not have any free cysteine residues. These muteins have been prepared as set forth in published PCT Publication No. WO 92/16221, specifically incorporated herein by reference, and the numbering is based upon the amino acid sequence set forth therein.

TABLE 2. MUTEINS OF 30kDa TNF INHIBITOR

c105 30kDa TNF inhibitor
c1 30kDa TNF inhibitor
c14 30kDa TNF inhibitor
c111 30kDa TNF inhibitor
c161 30kDa TNF inhibitor

The muteins and other polypeptides of the present invention include allelic variations in the protein sequence and substantially equivalent proteins. "Substantially equivalent," means possessing a very high degree of amino acid residue homology (See generally, M. Dayhoff, Atlas of Protein Sequence and Structure, vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by references) as well as possessing comparable biological activity. Also included within the scope of this invention are truncated forms of the native polypeptide or mutein that substantially retain the biological activity of the native polypeptide or mutein.

The conjugates of the present invention contain, in addition to biologically-active molecules having reactive thiol moieties, non-peptidic polymeric derivatives having active sulfone moieties. "Non-peptidic" means having less than 50% by weight of α amino acid residues.

The polymer portion of the polymeric derivative can be, for example, polyethylene glycol ("PEG"), polypropylene glycol ("PPG"), polyoxyethylated glycerol ("POG") and other polyoxyethylated polyols, polyvinyl alcohol ("PVA") and other polyalkylene oxides, polyoxyethylated sorbitol, or polyoxyethylated glucose. The polymer can be a homopolymer, a random or block copolymer, a terpolymer based on the monomers listed above, straight chain or branched, substituted or unsubstituted as long as it has at least one active sulfone

moiety. The polymeric portion can be of any length or molecular weight but these characteristics can affect the biological properties. Polymer average molecular weights particularly useful for decreasing clearance rates in pharmaceutical applications are in the range of 2,000 to 35,000 daltons. In addition, if two groups are linked to the polymer, one at each end, the length of the polymer can impact upon the effective distance, and other spatial relationships, between the two groups. Thus, one skilled in the art can vary the length of the polymer to optimize or confer the desired biological activity. If the polymer is a straight chain PEG, particularly useful lengths of polymers, represented by $(Z)_n$, where Z is the monomeric unit of the polymer, include n having a range of 50-500. In certain embodiments of the present invention, n is greater than 6 and preferably greater than 10.

Monomethoxy polyethylene glycol is designated here as mPEG. The term "PEG" means any of several condensation polymers of ethylene glycol. PEG is also known as polyoxyethylene, polyethylene oxide, polyglycol, and polyether glycol. PEG can also be prepared as copolymers of ethylene oxide and many other monomers. For many biological or biotechnical applications, substantially linear, straight-chain vinyl sulfone activated PEG will be used which is substantially unsubstituted except for the vinyl sulfone.

PEG is useful in biological applications for several reasons. PEG typically is clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze, and is nontoxic. PEGylation can improve pharmacokinetic performance of a molecule by increasing the molecule's apparent molecular weight. The increased apparent molecular weight reduces the rate of clearance from the body following subcutaneous or systemic administration. In many cases, PEGylation can decrease antigenicity and immunogenicity. In addition, PEGylation can increase the solubility of a biologically-active molecule.

The polymeric derivatives of the present invention have active sulfone moieties. "Active sulfone" means a sulfone group to which a two carbon group is bonded having a reactive site for thiol-specific coupling on the second carbon from the sulfone group at about pH 9 or less. Examples of active sulfones include, but are not limited to, vinyl sulfone and activated ethyl sulfone. An example of an active ethyl sulfone is $-SO_2-CH_2-CH_2-Z$ where Z is halogen or another leaving group capable of substitution by thiol to form the sulfone-thiol linkage $-SO_2-CH_2-CH_2-R$, where R represents a biologically active molecule. The sulfone-activated polymer can be further substituted as long as the thiol-specific reactivity at the second carbon is maintained at about pH 9 or less.

The sulfone-activated polymers of the present invention can be synthesized in at least four steps. Briefly, the first step is to increase the reactivity of a site on the polymer, typically an end group, by, for example, activation or substitution. The second step is to link sulfur directly to a carbon atom in the polymer in a form that can be converted to an ethyl sulfone or ethyl sulfone derivative having similar reactive properties. In the third step, the sulfur is oxidized to sulfone. In the fourth step, the second carbon from the sulfone group is activated.

The synthesis of a sulfone-activated polymer is described in more detail below using the synthesis of a sulfone-activated PEG as an example. The first step is the hydroxyl activation of an hydroxyl moiety in the PEG. The term "hydroxyl activation" should be interpreted herein to mean substitution as well as esterification and other methods of hydroxyl activation. Typically, in hydroxyl activation, an acid or an acid derivative such as an acid halide is reacted with the PEG to form a reactive ester in which the PEG and the acid moiety are linked through the ester linkage. The acid moiety generally is more reactive than the hydroxyl moiety. Typical esters are the sulfonate, carboxylate, and phosphate esters.

Sulfonyl acid halides that are suitable for use in the invention include, for example, methanesulfonyl chloride (also known as mesyl chloride) and *p*-toluene-sulfonyl chloride (also known as tosyl chloride). Methanesulfonate esters are sometimes referred to as mesylates. Toluenesulfonate esters are sometimes referred to as tosylates.

In a substitution type of hydroxyl activation, the entire hydroxyl group on the PEG is substituted by a more reactive moiety, typically a halide. For example, thionyl chloride, can be reacted with PEG to form a more reactive chlorine substituted PEG.

Thus, when PEG is the starting material, the typical reaction product of the first step is an ester or halide-substituted PEG.

In the second step, the ester or halide is substituted by an alcohol which contains a reactive thiol attached to an ethyl group, a thioethanol moiety. Thioethanol is an example of a suitable alcohol. In this step, the sulfur in the thiol is bonded directly to a carbon on the polymer.

Next, in the third step, the sulfur is oxidized to sulfone. Useful oxidizing agents include, for example, hydrogen peroxide, sodium perborate, or peroxy acids.

In the fourth step, the hydroxyl moiety of the alcohol used in step two is activated. This step is similar to the first step in the reaction sequence. Substitution typically is with halide to form a haloethyl sulfone or a derivative thereof having a reactive site on the second carbon removed from the sulfone moiety. Typically, the second carbon on the ethyl group will be activated by a chloride or bromide halogen. Hydroxyl activation should provide a site of similar reactivity, such as the sulfonate ester. Suitable reactants are, for example, the acids, acid halides, and others previously mentioned in discussing the first step in the reaction. Thionyl chloride is particularly useful for substitution of the hydroxyl group with the chlorine atom.

The resulting polymeric activated ethyl sulfone is stable, isolatable, and suitable for thiol-selective coupling reactions. PEG chloroethyl sulfone is stable in water at a pH of about 7 or less, but nevertheless can be used to advantage for thiol-selective coupling reactions at conditions of basic pH up to at least about pH 9. At a pH of above about 9, the thiol selectivity is diminished and the sulfone moiety becomes somewhat more reactive with amino groups. The linkage formed upon reaction with thiol is also hydrolytically stable.

In a fifth step that can be added to the synthesis, the activated ethyl sulfone is reacted with a base to from PEG vinyl sulfone or one of its active derivatives for thiol-selective coupling. Suitable bases include, for example, sodium hydroxide or triethylamine. Like activated ethyl sulfones, vinyl sulfone is hydrolytically stable, isolatable, thiol-selective, and forms hydrolytically-stable linkages upon reaction with thiol.

As used herein, "hydrolytically stable" means that the linkage between the polymer and the sulfone moiety and between the sulfone-thiol after conjugation does not react with water at a pH of less than about 11 for at least three days. Hydrolytic stability is desirable because, if the rate of hydrolysis is significant, the polymer can be deactivated before the reaction between polymer and the thiol of the biologically-active molecule takes place.

As mentioned above, for example, a linear PEG with active sites at each end will attach to a protein at one end, but, if the rate of hydrolysis is significant, will react with water at the other end to become capped with a relatively nonreactive hydroxyl moiety, rather than forming a "dumbbell" molecular structure with attached proteins or other desirable groups on each end. A similar problem arises when coupling a molecule to a surface by a PEG linking agent because the PEG is first attached to the surface or couples to the molecule, and the opposite end of the PEG derivative must remain active for a subsequent reaction. If hydrolysis is a problem, then the opposite end typically becomes inactivated.

Alternatively, the sulfone-activated derivatives can be prepared by attaching a linking agent having a sulfone moiety to a PEG (or other polymer) activated with a different functional group. For example, an amino activated PEG can be reacted under favorable conditions of pH of about 9 or less with a small molecule that has a succinimidyl active ester moiety at one terminus and vinyl sulfone at the other terminus. The amino-activated PEG forms a stable linkage with the succinimidyl ester. The resulting PEG is activated with the vinyl sulfone at the terminus and is hydrolytically stable: PEG-NH-OC-CH_2-CH_2-$SO_2CH{=}CH_2$.

A similar activated PEG can be achieved by reacting an amine-reactive PEG such as succinimidyl active ester PEG, PEG-CO_2-NHS, with a small molecule that has an amine moiety at one terminus and a vinyl sulfone moiety at the other terminus.

PEG chloroethyl sulfone and PEG vinyl sulfone were prepared as set forth in Example 1. Thiol-selective reactivity of PEG vinyl sulfone and chloroethyl sulfone is shown in Example 2. Hydrolytic stability of the polymer-sulfone linkage of two compounds is shown in Example 3. Hydrolytic stability of the linkage between thiol and sulfone is shown in Example 16.

When the polymer does not have an hydoxyl moiety, one can first be added by chemical methods well known in the art before carrying out the steps described above.

The activated polymeric derivatives of the present invention can have more than one reactive group. The derivatives can be monofunctional, bifunctional, or multifunctional. The reactive groups may be the same (homofunctional) or different (heterofunctional) as long as there is at least one active sulfone moiety.

Two particularly useful homobifunctional derivatives are PEG-*bis*-chlorosulfone and PEG-*bis*-vinyl sulfone. One skilled in the art can synthesize those molecules using PEG

having hydroxyl moieties at each end as a starting material and following the general method set forth above.

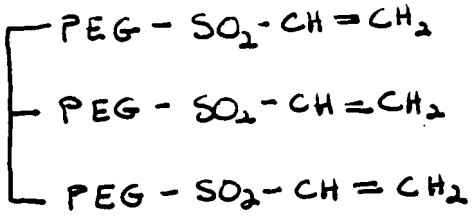
Heterobifunctional derivatives can also be synthesized. Two particularly useful heterobifunctional derivatives include, for example, a linear PEG with either a vinyl sulfone or a maleimide at one end and an N-hydroxysuccinimide ester ("NHS-ester") at the other end. The NHS-ester is amine-specific. PEG having an NHS-ester at one end and an activated sulfone moiety at the other can be attached to both lysine and cysteine residues. A stable amine linkage can be achieved, leaving the hydrolytically-stable unreacted sulfone available for subsequent reaction with thiol. Those two heterobifunctional PEG derivatives have been synthesized as described in Examples 5 and 6. If the maleimide NHS-ester heterobifunctional reagent is made using straight-chain PEG, represented by $(Z)_n$, where Z is the monomeric unit, n is greater than 6 and preferably greater than 10.

Other active groups for heterofunctional sulfone-activated PEGs can be selected from among a wide variety of compounds. For biological and biotechnical applications, the substituents would typically be selected from reactive moieties typically used in PEG chemistry to activate PEG such as the aldehydes, trifluoroethylsulfonate (sometimes called tresylate), n-hydroxylsuccinimide ester, cyanuric chloride, cyanuric fluoride, acyl azide, succinate, the *p*-diazo benzyl group, the 3-(*p*-diazophenyloxy)-2-hydroxy propyloxy group, and others.

Examples of active moieties other than sulfone are shown in Davis et al. U.S. Patent No. 4,179,337; Lee et al. U.S. Patent Nos. 4,296,097 and 4,430,260; Iwasaki et al. 4,670,417; Katre et al. U.S. Patent Nos. 4,766,106; 4,917,888; and 4,931,544; Nadagawa et al. U.S. Patent No. 4,791,192; Nitecki et al. U.S. Patent No. 4,902,502 and 5,089,261; Saifer U.S. Patent No. 5,080,891; Zalipsky U.S. Patent No. 5,122,614; Shadle et al. U.S.

Patent No. 5,153, 265; Rhee et al. U.S. Patent No. 5,162,430; European Patent Application Publication No. 0 247 860; and PCT International Application Nos. US86/01252; GB89/01261; GB89/01262; GB89/01263; US90/03252; US90/06843; US91/06103; US92/00432; and US92/02047, the contents of which are incorporated herein by reference.

An example of a trifunctional derivative is a glycerol backbone to which three vinyl sulfone PEG moieties are attached. This molecule can be represented by the formula:



This derivative was prepared as described in Example 12.

Another example of a mutifunctional derivative is the "star" molecule. Star molecules are generally described in Merrill U.S. Patent No. 5,171,264, incorporated herein by reference. Star molecules have a core structure to which multiple PEG chains or "arms" are attached. The sulfone moieties can be used to provide an active, functional group on the end of the PEG chain extending from the core and as a linker for joining a functional group or other moiety to the star molecule arms.

It should be apparent to the skilled artisan that the activated polymers discussed above could be used to carry a wide variety of substituents and combinations of substituents.

As stated above, the conjugates of the present invention are formed by reacting thiol-containing biologically-active molecules with sulfone-activated polymers. The linkage between the thiol reactive group and the sulfone-activated polymer is a covalent bond.

A general method for preparing the conjugates of the present invention includes the following steps:

(1) Choose the desired biologically-active molecule and determine if the molecule possesses a free thiol group by means well known in the art. See, for example, Allen, G., "Sequencing of Proteins and Peptides," pp. 153-54, in Laboratory Techniques in Biochemistry and Molecular Biology, Work, T.S., and Burdon, R.H., eds. (1972), incorporated herein by reference. If the molecule has a free thiol, proceed to step 3. If the molecule has no free thiol, proceed to step 2.

(2) If no free thiol exists in the molecule, add thiol as discussed above. After adding thiol, perform a bioassay to determine if the desired biological activity or a portion of the biological activity is retained.

(3) Synthesize the desired sulfone-activated polymer as discussed above.

(4) React the activated polymer with the molecule having a free thiol.

(5) Isolate the reaction product using chromatographic techniques well known in the art. For protein conjugates, see, for example, Scopes, R., Protein Purification, Cantor, C.R. ed., Springer-Verlag, New York (1982). For nonprotein molecules, see, for example, Still, W.C. et al., J. Org. Chem., 43, pp.2923-2925 (1978). If no conjugate forms, add thiol to another location on the biologically-active molecule and repeat steps (4) and (5).

(6) Determine biological activity of the conjugate formed using the relevant bioassay.

One skilled in the art can add or delete certain steps. For example, one skilled in the art might not assay bioactivity in step 2 or might presume biological activity after PEGylation based upon previous experiments. The skilled artisan can also add the step of varying the size, length, or molecular weight of the linker to optimize or confer biological activity.

Several conjugates have been prepared. The 30kDa TNFbp c105 mutein described above was conjugated with PEG vinyl sulfone as described in Example 10. Example 8 shows that native IL-1ra, which contains four free cysteines, reacted under similar conditions. The

c84 IL-1ra mutein also reacted well. Example 13 shows the conjugation of three 30kDa TNF inhibitor muteins to three PEG chains bonded to a glycerol backbone.

The conjugates of the present invention can be used for a variety of purposes including, but not limited to, in-vitro diagnostic assays and the preparation of pharmaceutical compositions. Many of the conjugates of the present invention have at least one of the following characteristics relative to the unconjugated molecule:

(1) increased solubility in aqueous solution;

(2) reduced antigenicity or immunogenicity;

(3) reduced rate of clearance following subcutaneous or systemic administration due to increased apparent molecular weight.

Pharmaceutical preparations of conjugates containing IL-1ra are particularly useful. IL-1ra, alone or in combination with the 30kDa TNF binding protein, can be used to treat arthritis, inflammatory bowel disease, septic shock, ischemia injury, reperfusion injury, osteoporosis, asthma, insulin diabetes, myelogenous and other leukemias, psoriasis, adult respiratory distress syndrome, cachexia/anorexia, and pulmonary fibrosis.

Conjugates containing TNF binding proteins ("TNFbps") are also particularly useful. Such conjugates can be used to treat TNF-mediated diseases such as adult respiratory distress syndrome, pulmonary fibrosis, arthritis, septic shock, inflammatory bowel disease, multiple sclerosis, graft rejection and hemorrhagic trauma.

The biologically active conjugates of the present invention can further include non-biologically active moieties.

The present invention also includes substantially purified compounds having the formula R_1-X-R_2, where at least one of R_1 and R_2 is a biologically-active molecule having a reactive thiol moiety which forms a covalent bond with X, a Michael acceptor-activated

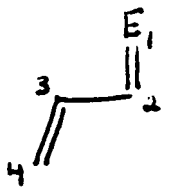
polymer. In the present invention, the biological activity of R_1-X-R_2 retains the biological activity of R_1 or R_2. Molecules having the formula R_1-X-R_2 are referred to herein as "dumbbell" molecules.

As stated above, the compounds of the present invention are substantially purified. "Substantially purified" as used herein means a "homogenous composition." A homogenous composition contains molecules of R_1-X-R_2 and is substantially free from compounds that (1) deviate in the composition of R_1 or R_2, or (2) are linked together by more than one activated polymer. The homogeneous composition can contain molecules of R_1-X-R_2 which differ in the length of X. For straight-chain polymers, represented by $(Z)_n$, where Z is the monomeric unit, n is greater than 6 and preferably greater than 10. To have a homogeneous composition, R_1 and R_2 need not be attached to X at the same location on X or on the same location on either R group.

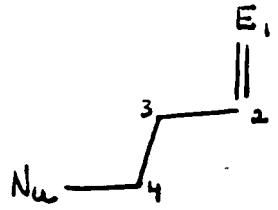
X is a non-peptidic polymer having a first reactive group and a second reactive group. A "reactive group" is a group capable of reacting with R. At least one reactive group on X is a Michael-type acceptor. The terms "reactive group" and "functional group" are used herein synonymously. The terms "Michael acceptor" and "Michael-type acceptor" are also used herein synonymously. Polymers suitable for use in the present invention are also discussed above and include, for example, PEG, POG, and PVA.

"Michael acceptors" are functional groups susceptible to Michael addition. "Michael addition" involves a nucleophilic attack on an electrophilic center which is adjacent to a pi system, having an electronegative atom. Examples of pi systems having an electronegative atom include sulfoxide, sulfonyl, carbonyl and heterocyclic aromatics. The nucleophile adds to the electrophilic center.

Michael acceptors can be represented by the formula:



where E is an electronegative atom. Addition takes place at the 4 position to form the following:



where N_u represents the nucleophile now bonded to the atom at position 4. Michael acceptor functional groups include, but are not limited to, maleimide and vinyl sulfone. The activated polymer from which a dumbbell is formed can, but need not, contain a vinyl sulfone species of Michael acceptor.

Activated polymers of the present invention include PEG having two or more Michael acceptor groups, including for example, PEG-*bis*-vinyl sulfone and PEG-*bis*-maleimide. PEG-*bis*-vinyl sulfone has been prepared as described in Examples 7. PEG-*bis*-maleimide has been prepared as described in PCT Publication No. WO 92/16221, incorporated herein by reference.

At least one of R_1 and R_2 is biologically active prior to coupling to X or to X-R. "Biologically active" has the same definition recited above. As stated above, biologically active molecules include, but are not limited to, binding proteins and targeting groups.

Both R_1 and R_2 can be biologically active but need not be. In some cases, if R_1 and R_2 have an affinity for the same ligand, the dumbbell can have a greater affinity for that ligand than either R_1 or R_2 alone. Published PCT Publication No. WO 92/16221 shows that the homodumbbell containing two molecules of 30kDa TNFbp linked by a PEG polymer is better at inhibiting cytotoxicity of TNFs in in-vitro assays than the 30kDa molecule alone.

In certain cases, R_1 can be a molecule which directs the compound R_1-X-R_2 to a certain location in a biological system and R2 can have an affinity for a ligand in that location.

Alternatively, only one of R_1 and R_2 can be biologically active in the compound R_1-X-R_2. The nonbiologically-active group can be a surface or any other biologically-inert molecule or compound.

In the present invention, the biologically active R group has a reactive thiol moiety. The biologically active R group can be a synthetic molecule. As used herein, the term "synthetic molecule" means a molecule to which a reactive thiol moiety has been added. Synthetic molecules include, for example, muteins containing a non-native cysteine. The thiol moiety reacts with a Michael-type acceptor of the polymer to form a covalent bond.

After formation of this covalent bond, the biologically-active molecule retains its biological activity. The R group "retains its biological activity" within the meaning of the invention if, after reaction with activated polymer, it has at least one tenth of the biological activity it had before reaction with polymer, preferably at least 40%, and more preferably at least 60%.

A general method for producing dumbbells follows:

(1) Choose an R group possessing the desired biological activity, for example, a protein such as tumor necrosis factor binding protein (TNFbp).

(2) Measure activity using the relevant bioassay.

(3) Determine the number of free sulfhydryl groups, for example, cysteine residues not involved in disulfide bonding, using generally known methods in the art. One such

method is described in Allen, G., "Sequencing of proteins and peptides," pp. 153-54, in Laboratory Techniques in Biochemistry and Molecular Biology, Work, T.S., and Burdon, R.H., eds. (1972). If there are no free cysteines, proceed to step 4(a). If there is one free cysteine, or only one accessible to the PEGylation reagent, proceed to the reaction step in 4(c). If the protein has more than one free cysteine, go to step 5.

(4) When R is polypeptide and no free cysteines exist:

(a) Create a mutein by inserting a cysteine or replacing a non-cysteine residue with a cysteine. Useful mutation sites include the N or C terminal ends of the protein, glycosylation sites, or lysine residues. Muteins can be routinely made, as stated above, by chemical synthesis or recombinant technology. Alternatively, chemically add a thiol moiety.

(b) Measure activity and compare that activity with the activity measured in step 2.

(c) If the mutein retains the activity measured in step 2, react the mutein with a polymer, such as PEG, having a single sulfhydryl-preferred reactive group. If the mutein bonds to the mono-reactive PEG (becomes PEGylated), measure activity and compare that activity with the activity measured in step 2. If the PEGylated mutein retains the activity measured in step 2, react the unPEGylated mutein with a PEG having two thiol-specific Michael Acceptors, such as *bis*-maleimide, to create dumbbell molecules. Repeat the bioassay to confirm that the dumbbells retain biological activity.

If one skilled in the art desires that R_1 and R_2 be different, the *bis*-reactive polymeric group can be reacted in series with R_1 and then R_2. Prior to reacting polymer with R_1, one of the two functional groups of the polymer is blocked or protected by means well known in

the chemical arts to form a protected group on X. See, for example, Greene, T.W. et al., Protective Groups in Organic Synthesis, John Wiley and Sons, Inc. (1991), incorporated herein by reference. In this context, "protected" means the functional group is not available for reaction. When X having a protected group is reacted with R_1, R_1-X, and not R_1-X-R_1, is formed. After R_1-X is formed, the blocking or protecting group is removed prior to reaction with R_2. "Deprotected" means the protective group is removed or the functional group is otherwise made available for reaction.

Alternatively, heterodumbbells can be formed by reacting R_1 with an excess of the *bis*-activated polymer to force R_1-X formation. After reaction, R_1-X is separated from the reaction mixture using chromatographic techniques well known in the art, including, for example, ion exchange chromatography. R_1-X is then reacted with R_2 to form R_1-X-R_2.

(d) If the mutein created in step 4(a) or the PEGylated mutein formed in step 4(c) does not substantially retain biological activity, start with the native protein, create a different mutein, and repeat steps 4(b) and 4(c). In addition, the length or molecular weight of the polymer X can be changed to optimize or confer biological activity.

(5) For proteins with more than one free cysteine, monoPEGylate, bioassay, and react with the bifunctional PEGylation reagent. If higher-ordered structures are formed, i.e. more than two proteins are PEG-linked, separate the dumbbells via chromatographic methods known in the art. Where such separation is undesirable for any reason, delete or replace a free cysteine with another amino acid and proceed to step 4 (b).

(6) For non-protein biologically-active R groups, exploit free sulfhydryl groups for attachment to the polymer X. Add free sulfhydryl groups to the molecule if necessary or desirable.

One skilled in the art might choose to modify, add or delete certain steps. For example, one might choose to react active proteins with a bifunctional-PEG and skip the monoPEGylation step.

Several dumbbell molecules of the present invention have been prepared. Published PCT Application No. WO 92/16221, which is incorporated herein by reference, sets forth the preparation of the following dumbbells prepared using *bis*-maleimido-PEG: 30kDa TNF inhibitor homodumbbells, Il-2 inhibitor heterodumbbell, heterodumbbells which inhibit the classical pathway of the complement system, and IL-1ra and PDGF heterodumbbells.

Pharmaceutical compositions containing many of the conjugates or compounds (collectively, the "conjugates") of the present invention can be prepared. These conjugates can be in a pharmaceutically-acceptable carrier to form the pharmaceutical compositions of the present invention. The term "pharmaceutically acceptable carrier" as used herein means a non-toxic, generally inert vehicle for the active ingredient, which does not adversely affect the ingredient or the patient to whom the composition is administered. Suitable vehicles or carriers can be found in standard pharmaceutical texts, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980), incorporated herein by reference. Such carriers include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline. In addition, the carrier can contain other pharmaceutically-acceptable excipients for modifying

or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation.

The pharmaceutical compositions can be prepared by methods known in the art, including, by way of an example, the simple mixing of reagents. Those skilled in the art will know that the choice of the pharmaceutical carrier and the appropriate preparation of the composition depend on the intended use and mode of administration.

In one embodiment, it is envisioned that the carrier and the conjugate constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier can be either aqueous or non-aqueous in nature. In addition, the carrier can contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier can contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the conjugate. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations can be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing the conjugates are stored and administered at or near physiological pH. It is presently believed that administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The manner of administering the formulations containing the conjugates for systemic delivery can be via subcutaneous, intramuscular, intravenous, oral, intranasal, or vaginal or rectal suppository. Preferably the manner of administration of the formulations containing the conjugates for local delivery is via intraarticular, intratracheal, or instillation or inhalations to the respiratory tract. In addition it may be desirable to administer the conjugates to specified portions of the alimentary canal either by oral administration of the conjugates in an appropriate formulation or device.

In another suitable mode for the treatment of osteoporosis and other bone loss diseases, for example, an initial intravenous bolus injection of TNF inhibitor conjugate and IL-1 inhibitor conjugate is administered followed by a continuous intravenous infusion of TNF inhibitor conjugate and IL-1 inhibitor conjugate. For oral administration, the conjugate is encapsulated. The encapsulated conjugate can be formulated with or without pharmaceutically-acceptable carriers customarily used in the compounding of solid dosage forms. Preferably, the capsule is designed so that the active portion of the formulation is released at that point in the gastro-intestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients can be included to facilitate absorption of the conjugate. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, route of administration and the age, sex and medical condition of the pateint. In certain embodiments, the dosage and administration is designed to create a preselected concentration range of the conjugate in the patient's blood stream. For example, it is believed that the maintenance of circulating

concentrations of TNF inhibitor and IL-1 inhibitor of less than 0.01 ng per mL of plasma may not be an effective composition, while the prolonged maintenance of circulating levels in excess of 10 μg per mL may have undesirable side effects. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data.

It should be noted that the conjugate formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

The following examples are illustrative of the invention and are not intended as limitations.

Example 1: Synthesis

The reaction steps can be illustrated structurally as follows:

(1) $PEG\text{-}OH + CH_3SO_2Cl \rightarrow PEG\text{-}OSO_2CH_3$

(2) $PEG\text{-}OSO_2CH_3 + HSCH_2CH_2OH \rightarrow PEG\text{-}SCH_2CH_2OH$

(3) $PEG\text{-}SCH_2CH_2OH + H_2O_2 \rightarrow PEG\text{-}SO_2CH_2CH_2OH$

(4) $PEG\text{-}SO_2CH_2CH_2OH + SOCl_2 \rightarrow PEG\text{-}SO_2CH_2CH_2Cl$

(5) $PEG\text{-}SO_2CH_2CH_2Cl + NaOH \rightarrow PEG\text{-}SO_2\text{-}CH{=}CH_2 + HCl$

Each of the above reactions is described in detail below:

Reaction 1. Reaction 1 represents the preparation of the methane sulfonyl ester of polyethylene glycol, which can also be referred to as the methanesulfonate or mesylate of polyethylene glycol. The tosylate and the halides can be prepared by similar procedures, which are believed to be apparent to the skilled artisan.

To prepare the mesylate, twenty-five grams of PEG of molecular weight 3400 was dried by azeotropic distillation in 150 mL of toluene. Approximately half of the toluene was distilled off in drying the PEG. Forty mL of dry dichloromethane was added to the toluene and PEG solution, followed by cooling in an ice bath. To the cooled solution was added 1.23 mL of distilled methanesulfonyl chloride, which is an equivalent weight of 1.6 with respect to PEG hydroxyl groups, and 2.66 mL of dry triethylamine, which is an equivalent weight of 1.3 with respect to PEG hydroxyl groups. "Equivalent weight" as used above can be thought of as "combining weight" and refers to the weight of a compound that will react with an equivalent weight of PEG hydroxyl groups.

The reaction was permitted to run overnight during which time it warmed to room temperature. Triethylammonium hydrochloride precipitated and the precipitate was removed by filtration. Thereafter, the volume was reduced by rotary evaporation to 20 mL. The mesylate was precipitated by addition to 100 mL of cold dry ethyl ether. Nuclear magnetic resonance (NMR) analysis showed 100% conversion of hydroxyl groups to mesylate groups.

Reaction 2. Reaction 2 represents the formation of polyethylene glycol mercaptoethanol by reaction of the mesylate with mercaptoethanol. The reaction causes the methanesulfonate radical to be displaced from the PEG. The sulfur in the mercaptoethanol radical is attached directly to the carbon in the carbon-carbon backbone of the PEG.

Twenty grams of the mesylate from reaction 1 was dissolved in 150 mL of distilled water. The solution of mesylate and water was cooled by immersion in an ice bath. To the cooled solution was added 2.37 mL of mercaptoethanol, which is 3 equivalent weights with respect to PEG hydroxyl groups. Also added was 16.86 mL of 2N NaOH base. The reaction was refluxed for 3 hours, which means that the vapors rising from the heated reaction were continuously condensed and allowed to flow back into the reaction.

The polyethylene glycol mercaptoethanol product was extracted three times with dichloromethane using approximately 25 mL of dichloromethane each time. The organic fractions were collected and dried over anhydrous magnesium sulfate. The volume was reduced to 20 mL and the product was precipitated by addition to 150 mL of cold dry ether.

NMR analysis in d_6-DMSO (dimethyl sulfoxide) gave the following peaks for PEG-SCH_2CH_2OH: 2.57 ppm, triplet, -CH_2-S-; 2.65 ppm, triplet, -S-CH_2-; 3.5 ppm, backbone singlet; and 4.76 ppm, triplet, -OH. Integration of the peak for -S-CH_2- indicated 100% substitution.

Reaction 3. Reaction 3 represents peroxide oxidation of the polyethylene glycol mercaptoethanol product to convert the sulfur, S, to sulfone, SO_2. PEG-β-hydroxysulfone is produced.

Twenty grams of PEG-SCH_2CH_2OH was dissolved in 30 mL of 0.123M tungstic acid solution and cooled in an ice bath. The tungstic acid solution was prepared by dissolving the acid in sodium hydroxide solution of pH 11.5 and then adjusting the pH to 5.6 with glacial acetic acid. Twenty mL of distilled water and 2.88 mL of 30% hydrogen peroxide, which has an equivalent weight of 2.5 with respect to hydroxyl groups, was added to the solution of tungstic acid and polyethylene glycol mercaptoethanol and the reaction was permitted to warm overnight to room temperature.

The oxidized product was extracted three times with dichloromethane using 25 mL of dichloromethane each time. The collected organic fractions were washed with dilute aqueous sodium bicarbonate and dried with anhydrous magnesium sulfate. The volume was reduced to 20 mL. The PEG-β-hydroxysulfone product was precipitated by addition to cold dry ethyl ether.

NMR analysis in d_6-DMSO gave the following peaks for PEG-SCH_2CH_2OH: 3.25 ppm, triplet, -CH_2-SO_2-; 3.37 ppm, triplet, -SO_2-CH_2-; 3.50 ppm, backbone; 3.77 ppm, triplet, -CH_2OH; 5.04 ppm, triplet, -OH. The hydroxyl peak at 5.04 ppm indicated 85% substitution. However, the peak at 3.37 ppm for -SO_2-CH_2- indicated 100% substitution and is considered to be more reliable.

Reaction 4. Reaction 4 represents the final step in synthesis, isolation, and characterization of polyethylene glycol chloroethyl sulfone.

To synthesize the product, twenty grams of PEG-$SO_2CH_2CH_2OH$, PEG-β-hydroxysulfone, was dissolved in 100 mL of freshly distilled thionyl chloride and the solution was refluxed overnight. The thionyl chloride had been distilled over quinoline. Excess thionyl chloride was removed by distillation. Fifty mL of toluene and 50 mL of dichloromethane were added and removed by distillation.

To isolate the product, the PEG chloroethyl sulfone was dissolved in 20 mL of dichloromethane and precipitated by addition to 100 mL of cold dry ethyl ether. The precipitate was recrystallized from 50 mL of ethyl acetate to isolate the product.

Nuclear magnetic resonance was used to characterize the product. NMR analysis of PEG-$SO_2CH_2CH_2Cl$ in d_6-DMSO gave the following peaks: 3.50 ppm, backbone; 3.64 ppm, triplet, -CH_2SO_2-; 3.80 ppm, triplet, -SO_2-CH_2-. A small hydroxyl impurity triplet

appeared at 3.94 ppm. Calculation of the percentage substitution was difficult for this spectrum because of the proximity of the important peaks to the very large backbone peak.

Reaction 5. Reaction 5 represents conversion of polyethylene glycol chloroethyl sulfone from reaction step 4 to polyethylene glycol vinyl sulfone and isolation and characterization of the vinyl sulfone product.

The PEG vinyl sulfone was readily prepared by dissolving solid PEG chloroethyl sulfone in dichloromethane solvent followed by addition of two equivalents of NaOH base. The solution was filtered to remove the base and the solvent was evaporated to isolate the final product PEG-SO_2-CH=CH_2, PEG vinyl sulfone.

The PEG vinyl sulfone was characterized by NMR analysis in d_6-DMSO dimethyl sulfoxide. NMR analysis showed the following peaks: 3.50 ppm, backbone; 3.73 ppm, triplet, -CH_2-SO_2-; 6.21 ppm, triplet, =CH_2; 6.97 ppm, doublet of doublets, -SO_2-CH-. The 6.97 ppm peak for -SO_2-CH- indicated 84% substitution. The 6.21 ppm peak for =CH_2 indicated 94% substitution. Titration with mercaptoethanol and 2,2'-dithiodipyridine indicated 95% substitution.

Example 2: Thiol-selective Reactivity

Example 2 shows that PEG vinyl sulfone and its precursor PEG chloroethyl sulfone are significantly more reactive with thiol groups (-SH) than with amino groups (-NH_2) or imino groups (-NH-). Compounds containing thiol groups are organic compounds that resemble alcohols, which contain the hydroxyl group -OH, except that in thiols, the oxygen of the hydroxyl group is replaced by sulfur. Thiols sometimes are also called sulfhydryls or mercaptans. PEG vinyl sulfone contains the vinyl sulfone group -SO_2-CH=CH_2. PEG chloroethyl sulfone contains the chloroethyl sulfone group -$SO_2CH_2CH_2Cl$.

Selectivity for thiols is important in protein modification because it means that cysteine units (containing -SH) will be modified in preference to lysine units (containing -NH_2) and histidine units (containing -NH-). The selectivity of PEG vinyl sulfone for thiols means that PEG can be selectively attached to cysteine units, thus preserving protein activity for specific proteins and controlling the number of PEG molecules attached to the protein.

The relative reactivity of PEG vinyl sulfone with thiol and amino groups was determined by measuring the rates of reaction of PEG vinyl sulfone with N-α-acetyl lysine methyl ester and with mercaptoethanol. N-α-acetyl lysine methyl ester is a lysine model containing an amino group and is abbreviated Lys-NH_2. Mercaptoethanol serves as a cysteine model containing a thiol group and is abbreviated Cys-SH. Relative reactivity of PEG chloroethyl sulfone was similarly determined. This molecule may serve as a "protected" form of the vinyl sulfone since it is stable in acid but converts to PEG vinyl sulfone upon addition of base.

Reactivity for PEG vinyl sulfone and for the PEG chloroethyl sulfone precursor was investigated at pH 8.0, pH 9.0, and at pH 9.5. Buffers for controlling the pH were 0.1 M phosphate at pH 8.0 and 0.1 M borate at pH 9.0 and at pH 9.5. For measurement of mercaptoethanol reactivity, 5 mM ethylenediamine tetraacetic acid (EDTA) was added to both buffers to retard conversion of thiol to disulfide.

For reaction of the PEG derivatives of the invention with Lys-NH_2, a 3 mM solution of the PEG derivative was added under stirring to a 0.3 mM Lys-NH_2 solution in the appropriate buffer for each of the three levels of basic pH. The reaction was monitored by addition of fluorescamine to the reaction solution to produce a fluorescent derivative from reaction with remaining amino groups. The monitoring step was performed by adding 50 μL of reaction to 1.95 mL of phosphate buffer of pH 8.0 followed by adding 1.0 mL of

fluorescamine solution under vigorous stirring. The fluorescamine solution was 0.3 mg fluorescamine per mL of acetone.

Fluorescence was measured 10 minutes after mixing. Excitation was at wavelength 390 nm. Light emission occurred at 475 nm. No reaction was observed in 24 hours for either PEG vinyl sulfone or PEG chloroethyl sulfone at pH 8.0. At pH 9.5 the reaction was slow, but all amino groups were reacted after several days.

For reaction of the PEG vinyl sulfone and PEG chloroethyl sulfone precursor with Cys-SH, a 2 mM solution of the PEG derivative was added to a 0.2 mM solution of Cys-SH in the appropriate buffer for each of the three levels of basic pH. The reaction was monitored by adding 4-dithiopyridine to the reaction solution. The 4-dithiopyridine compound reacts with Cys-SH to produce 4-thiopyridone, which absorbs ultraviolet light.

The monitoring step was performed by adding 50μL of reaction mixture to 0.95 mL of 0.1 M phosphate buffer at pH 8.0 and containing 5 mM EDTA, followed by adding one mL of 2 mM 4-dithiopyridine in the same buffer.

Absorbance of 4-thiopyridone was measured at 324 nm. Both PEG vinyl sulfone and PEG chloroethyl sulfone showed reactivity toward Cys-SH, with PEG vinyl sulfone showing greater reactivity. At pH 9.0 the reaction is over within two minutes using the vinyl sulfone and within 15 minutes using the chloroethyl sulfone. However, these reactions were too fast for determination of accurate rate constants. At pH 8.0 the reactions were slower, but still complete in one hour for vinyl sulfone and in three hours for the chloroethyl sulfone. The conversion of chloroethyl sulfone to vinyl sulfone is significantly slower than the reaction of vinyl sulfone with Cys-SH. Thus the rate of reaction for chloroethyl sulfone with Cys-SH appears to be dependent on the rate of conversion of

chloroethyl sulfone to vinyl sulfone. Nevertheless, these reaction rates were still much faster than for the reaction with Lys-NH_2.

The above kinetic studies demonstrate the following points. PEG vinyl sulfone is much more reactive with thiol groups than with amino groups, indicating that attachment of PEG vinyl sulfone to a protein containing both cysteine and lysine groups proceeds primarily by reaction with cysteine. Since reactivity with amino groups is similar to imino groups, then reactivity of histidine subunits will also be much lower than reactivity with cysteine subunits. Also, selectivity toward thiol groups is accentuated at lower pH values for PEG chloroethyl sulfone and PEG vinyl sulfone, although the reactions of PEG chloroethyl sulfone are somewhat slower.

The utility of many PEG derivatives is limited because they react rapidly with water, thus interfering with attempts to attach the derivative to molecules and surfaces under aqueous conditions. The following Example 3 shows that PEG vinyl sulfone and PEG chloroethyl sulfone are stable in water.

Example 3: Hydrolytic Stability

PEG vinyl sulfone was dissolved in heavy water, D_20 deuterium oxide, and monitored by NMR. Reaction did not occur. A solution of PEG chloroethyl sulfone produced PEG vinyl sulfone in heavy water that was buffered with borate to pH 9.0. Monitoring with NMR showed that PEG vinyl sulfone, once produced, was stable for three days in heavy water.

PEG chloroethyl sulfone is stable in water until solution becomes basic, at which time it is converted into vinyl sulfone. Conversion to vinyl sulfone has been demonstrated by dissolving PEG chloroethyl sulfone in water at pH 7 and in borate buffer at

pH 9. The PEG derivative is extracted into methylene chloride. Removal of methylene chloride followed by NMR analysis showed that PEG chloroethyl sulfone is stable at a neutral pH of 7.0, and reacts with base to produce PEG vinyl sulfone.

Vinyl sulfone is stable for several days in water, even at basic pH. Extensive hydrolytic stability and thiol-specific reactivity of PEG vinyl sulfone means that PEG vinyl sulfone and its precursor are useful for modification of molecules and surfaces under aqueous conditions, as shown in the following Example 4.

Example 4: Conjugation to BSA

Protein modification was demonstrated by attachment of the PEG derivative to bovine serum albumin (BSA) by two different methods. BSA is a protein. Native unmodified BSA contains cystine groups which do not contain thiol groups. The cystine units are tied up as disulfide linkages, S-S.

In the first method, m-PEG (monomethoxy-PEG) vinyl sulfone of molecular weight 5,000 was reacted with unmodified BSA for 24 hours in a 0.1 M borate buffer at pH 9.5 at room temperature. The solution contained 1 mg of BSA and 1 mg of m-PEG vinyl sulfone, of molecular weight 5,000, per mL of solution. The results from the Example 2 model compounds had indicated that lysine subunits (and possibly histidine subunits) would be modified under these relatively basic conditions and in the absence of free thiol groups available for reaction.

Attachment to lysine subunits was demonstrated in two ways. First, size exclusion chromatography showed that the molecular weight of the protein had increased by approximately 50%, thus indicating attachment of approximately 10 PEGs to the protein.

Second, fluorescamine analysis showed that the number of lysine groups in the BSA molecule had been reduced by approximately ten.

In the second method, the BSA was treated with tributylphosphine to reduce the disulfide S-S bonds to thiol groups, -SH, which are available for reaction. The modified BSA was then treated with PEG chloroethyl sulfone at pH 8.0 in a 0.1 M phosphate buffer at room temperature for 1 hour. The solution contained 1 mg of modified BSA and 1 mg of m-PEG chloroethyl sulfone of molecular weight 5,000 per mL of solution. The results showed that lysine groups were unreactive under these conditions. However, thiol groups were reactive.

Attachment of the PEG to the protein was demonstrated by size exclusion chromatography, which showed an increase in the molecular weight of the protein by about 25%. Fluorescamine analysis indicated no change in number of lysine subunits in the protein, thus confirming that PEG attachment did not take place on lysine subunits. Substitution on thiol groups was thereby confirmed.

EXAMPLE 5: Synthesis of vinyl sulfone NHS-ester heterobifunctional PEG (3,400) reagent.

Briefly, PEG(3,400)-ω-vinyl sulfone-α-priopionic acid, succinimidyl ester was synthesized in several steps. First, the ethyl ester of PEG(3,400)-ω-hydroxy-α-propionic acid was synthesized. Second, the ethyl ester was converted to the ω-mesylate derivative. Third, the mesylate was used to prepare the ω-thioethanol derivative. Fourth, the thioethanol derivative was converted to the ω-hydroxysulfone. Fifth, the hyroxysulfone was converted to the ω-vinyl sulfone. The latter α-ethyl ester was converted to the α-propionic acid in a

sixth step. Finally, the propionic acid group was converted to the succinimidyl ester. The detailed synthesis is set forth below.

Step 1. 15.0 grams of PEG(3,400)-ω-hydroxy-α-propionic acid, 75 mL anyhydrous ethyl alcohol, and 3 mL sulfuric acid were heated to reflux for 1 hour. After cooling to room temperature, 50 mL water was added to the reaction mixture and sodium bicarbonate was used to adjust pH to 7. Ethyl alcohol was distilled off under reduced pressure using a rotoevaporator at 55°C for one-half hour. The reaction product was extracted with 60, 50 and 40 mL dichloromethane. The extract was dried with anhydrous magnesium sulfate, concentrated to 50 mL, and added to 400 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield of the ethyl ester was 13.1 grams. NMR analysis showed 49% propionic acid, ethyl ester groups and 51% PEG-OH groups.

Step 2. A mixture of 13.0 grams (0.0038 mol) of the ethyl ester derivative formed in step 1, 100 mL toluene, and 2.0 grams BHT was azeotropically dried during heating to reflux. Next, 15 mL dry dichloromethane, 0.60 mL (0.0043 mol, 1.15 fold excess) triethylamine and 0.31 mL (0.0040 mol, 1.07 fold excess) mesyl chloride were added at 5°C and the mixture was stirred overnight at room temperature under a nitrogen atmosphere. 2 mL anhydrous ethyl alcohol was added and the mixture was stirred for 15 minutes. The mixture was then filtered and about 70 mL of solvents were distilled off under reduced pressure to yield a toluene solution of PEG-ω-mesylate-α-propionic acid ethyl ester.

Step 3. The following were added to about 40 mL (0.00375 mol) of the PEG-ω-mesylate-α-propionic acid ethyl ester solution obtained in step 2: 150 mL of anyhydrous ethyl alcohol, 1.79 mL (0.0139 mol, 3.69 fold excess) mercaptoethanol and 0.45 grams (0.0011 mol, 3.0 fold excess) sodium hydroxide dissolved in 20 mL anhydrous ethyl alcohol. The

mixture was heated 3 hours at 58-62°C under a nitrogen atmosphere. After cooling to room temperature, acetic acid was used to adjust the pH to about 6.5 and 140 mL of ethyl alcohol was distilled off under reduced pressure using a rotoevaporator, at 55°C for 40 minutes. After distillation, 50 mL dichloromethane was added to the residue. The resulting solution was washed with distilled water and dried with anhydrous magnesium sulfate. The solution was then concentrated to 30 mL and added to 350 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield of the thioethanol derivative was 11.5 grams. NMR analysis showed 52% thioethanol groups, 35% propionic acid, ethyl ester groups and 13% PEG-OH moieties.

Step 4. Next, a solution of 11.5 grams PEG-ω-thioethanol-α-propionic acid, ethyl ester in 12 mL distilled water was prepared. A tungstic acid solution was also prepared as follows: 0.14 grams of tungstic acid, 12.0 mL distilled water and 0.05 grams sodium hydroxide dissolved in 6.0 mL water were mixed to form a solution having a pH of 11.5. A 10% solution of NaH_2PO_4 was added to the tungstic acid solution to adjust the pH to 6.6. The 12 mL solution of ethyl ester was then added to the pH 6.6 tungstic acid solution and the pH was again adjusted to 6.6 with 0.1M NaOH. 1.1 mL of 30% hydrogen peroxide was added and the reaction mixture was stirred for 19 hours. The pH after the reaction period was 6.7. 1M NaOH was added to adjust the pH to 7.2 and the reaction mixture was stirred for 1 hour. 5 grams of sodium chloride dissolved in 45 mL distilled water was added to the reaction mixture. The reaction product was extracted 3 times with 50 mL dichloromethane. The extract was dried with magnesium sulfate as follows: 10 grams powdered magnesium sulfate was added to the extract and the magnesium sulfate was filtered away after two hours. The magnesium sulfate dried extract was concentrated to 40 mL and added to 350 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure.

The yield was 9.7 grams and contained 50% hydroxysulfone groups, 39% propionic acid, ethyl ester groups and 11% PEG-OH groups as determined by NMR. **Step 5.** To a mixture of: 9.6 grams (0.00271 mol) of the PEG-ω-hydroxysulfone-α-propionic acid, ethyl ester synthesized in step 4, 50 mL dichloromethane and 0.01 grams (0.1 wt % per PEG) BHT stirred at room temperature under a nitrogen atmosphere was added 3.00 mL (0.0215 mol, 3.97 fold excess) triethylamine and 0.80 mL (0.010 mol, 3.81 fold excess) mesyl chloride. The reaction mixture was stirred for 15 minutes, filtered, and diluted with 150 mL dichloromethane. The resulting mixture was then washed with 25 mL 1M HCl, 25 mL 10% NaCl and 25 mL water. A small amount of Na_2HPO_4 was added to adjust the pH of the water layer to 7. The reaction mixture was then dried with magnesium sulfate and concentrated to 40 mL. The obtained solution was added to 400 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure to yield 9.1 grams. NMR analysis showed the following functionalities: 43% vinyl sulfone, 16% mesylate, and 35% propionic acid, ethyl ester.

Step 6. To a solution of 9.0 grams of the PEG-ω-vinyl sulfone-α propionic acid, ethyl ester derivative in 50 mL distilled water, 1.0M NaOH was added to adjust the pH to 12.0 and the solution was stirred 1.5 hours keeping the pH between 11.9 and 12.1 by periodic addition of 1.0M NaOH. Next, the pH was adjusted to 3.0 with oxalic acid, 5 grams of NaCl was added to the solution, and the reaction product was extracted 3 times with 50 mL dichloromethane. The extract was dried with anhydrous magnesium sulfate, concentrated to 30 mL and added to 350 mL cold diethyl ether. The precipitate was filtered off and dried under reduced pressure. The yield was 6.8 grams. Functional groups identified by NMR analysis were: vinyl sulfone 40%, propionic acid 29%, propionic acid, ethyl ester 4%, and 17% mesylate. The precipitate was purified by ion-exchange chromatography over a DEAE

Sepharose FF column. The yield after purification was 3.2 grams and NMR analysis showed 50% propionic acid groups, 38% vinyl sulfone groups, and 8% mesylate groups.

Step 7. A mixture of 3.0 grams PEG-ω-vinyl sulfone-α-propionic acid, 0.12 grams N-hydroxysuccinimide, 0.21 grams DCC (dicyclohexylcarbodiimide) in 20 mL dichloromethane was stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was then filtered and added to 250 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure to yield 2.90 grams. NMR showed the following groups: succinimide 50%, 38% vinyl sulfone, 10% mesylate, and 2% hydroxysulfone.

EXAMPLE 6: Synthesis of maleimide, NHS-ester heterobifunctional PEG (3,400) reagent.

The maleimide, NHS-ester PEG reagent was synthesized in two steps. In the first step, maleimido-PEG-OH was synthesized. Specifically, 0. 130 grams maleimido succinimidyl propionate were dissolved in 5 mL dry dichloromethane and cooled to 0°C. Next, 0.5 grams PEG-monoamine, prepared as described below, was added and then 2 drops of triethylamine. After 2 hours at room temperature, TLC indicated that the reaction was complete. TLC was conducted using n-BUOH-ACOH-H_2O at a ratio of 4:1:1. The reaction mixture was evaporated to dryness and the residue dissolved in 15 mL distilled water. The pH of the solution was adjusted to 3 using 15 mL 0.5M HCl and extracted with 10 mL CH_2Cl_2. The organic layer was dried with magnesium sulfate, filtered, concentrated to 15 mL, and poured into 75 mL cold ether. The precipitate was filtered and dried in vacuo. The yield was 0.300 grams. NMR analysis showed 77% maleimide groups and 100% PEG-OH.

In the second step, the maleimido-PEG-OH was converted to the maleimide-PEG-NHS-ester. A mixture of 2 mL CH_2Cl_2, 0.05 mL pyridine (1 equivalent) 1 mL acetonitrile and 0.266 grams maleimido-PEG-OH was stirred at room temperature under nitrogen. To this mixture, 0.070 grams (2.5 equivalents) N,N-disuccinimidyl carbonate was added and the reaction left overnight. The reaction mixture was then poured into approximately 50 mL cold ether, filtered and dried in vacuo. The NMR showed impurities and the product was precipitated a second time with a final yield of 0.230 grams.

The PEG-monoamine used in the first step above was prepared in three steps as follows. First, the PEG-mesylate derivative was formed. From the mesylate, the amine was formed. Finally, the monoamine was separated from the underivatized PEG and the diamine.

Step 1 PEG-3,400 (120 grams, 0.07164 equivalents of OH) was dissolved in 580 mL toluene, azeotropically dried, and then 90 mL dichloromethane, 1.80 mL triethylamine (0.01291 mol) and 0.83 mL mesyl chloride (0.01072 mol) were added. After overnight reaction at room temperature, 90 mL of solvents were distilled off from the reaction mixture under reduced pressure, the mixture was filtered and then 500 mL toluene was distilled off under reduced pressure. The residue was added to 800 mL cold diethyl ether. The precipitated product was filtered off and dried under reduced pressure. The yield was 118 grams and the substitution was 15%.

Step 2 118 grams of the mesylate formed in step 1 and 80 grams ammonium chloride were dissolved in 1600 mL concentrated aqueous NH_4OH and stirred at room temperature for 44 hours. The reaction product was extracted with 600, 400, and then 200 mL dichloromethane. The extract was washed with 170 mL 2% KOH and 170 mL water, dried with magnesium sulfate, concentrated to 200 mL and added to 800 mL cold diethyl ether.

The precipitated product was filtered off and dried under reduced pressure. The yield was 106 grams and the substitution was 15.6%.

Step 3 45 grams of the amine formed in step 2 was dissolved in 9 L water and loaded onto SP-Sepharose FF (300 mL of gel equilibrated with 1000 mL citric acid-lithium citrate buffer, 0.4%, pH 3.0, and then washed with water). SP-Sepharose FF is available from Pharmacia, Uppsala, Sweden. The underivatized PEG was washed off the column with water. Next, PEG monoamine was eluted with 800 mL 20 mM NaCl. The pH of the eluate was adjusted to 11 with 1M NaOH and the PEG monoamine was extracted with dichloromethane, dried with magnesium sulfate, and the solvent was distilled off. The yield was 9 grams.

EXAMPLE 7: SYNTHESIS OF PEG-α,ω-*bis*-vinyl sulfone.

The synthesis of 3,400 and 20,000 kDa PEG *bis*-vinyl sulfone was conducted using PEG diol and the general method set forth above. PEG diol was purchased from Fluka Chemical Corporation (Ronkonkoma, New York) or from Nippon Oil and Fat (Tokyo, Japan).

EXAMPLE 8: PEGYLATION of IL-1ra using PEG-20,000 -α,ω-*bis*-vinyl sulfone.

The IL-1ra c84 mutein was prepared as set forth in published PCT Application WO 92/16221, incorporated herein by reference. Conjugation of the c84 mutein or the native (wild-type) IL-1ra using PEG-α,ω-*bis*-vinyl sulfone (3,400 or 20,000 kDa) was conducted at 25°C in citrate buffer, pH 6.75-7.5, in 1 mL tubes, varying PEG and protein concentrations. At a protein concentration of 30 mg/mL, good conversion to the dumbbell molecule was obtained within 18 hours. At a protein concentration of 0.94 mg/mL, mostly monoadducts

were obtained. The dumbbell species was preferentially formed at a protein concentration of 100 mg/mL with 0.03 equivalents PEG. The dumbbell can be purified using chromatographic techniques set forth in PCT Publication Publication No. WO 92/16221, incorporated herein by reference.

In other experiments 0.1 M Tris-HCl buffer, pH 8.5, containing 30 mg/mL of the wild-type IL-1ra was treated with a 0.53 molar equivalent of the 20kDa PEG-*bis*-vinyl sulfone at 25°C for 18 hours. SDS PAGE analysis showed conversion to both dumbbell and the monoadduct. At a protein concentration of 3.1 mg/mL with 1 molar equivalent of PEG reagent, only the monoadduct was observed.

In general, the c84 mutein reacts more readily with the PEG reagent than the wild-type molecule.

EXAMPLE 9. Bioactivity of IL-1ra dumbbell.

The c84 dumbbell generated above was analyzed for its receptor binding affinity compared to that of unPEGylated recombinant IL-1ra on murine EL-4 cells using the assay set forth in PCT Application Publication No. WO 92/16221, incorporated herein by reference. The results showed similar binding affinities between the two molecules.

EXAMPLE 10: PEGYLATION of TNFbp c105 mutein with PEG-20,000-α, ω-*bis*-vinyl sulfone.

The c105 mutein of TNFbp was prepared as set forth in published PCT Publication WO 92/16221, incorporated herein by reference. Alternatively, the c105 mutein was prepared as follows. E. Coli cells expressing the c105 mutein were harvested by centifugation. The cell sludge was adjusted to approximately 40% wet weight solids by the

addition of purified water. The mixture was then further diluted with an equal volume of breaking buffer (50 mM Tromethamine, 4 mM EDTA, pH 7.2) to give a suspension with approximately 20% wet weight solids. The cell sludge was passed five times through a high pressure homogenizer operating at approximately 8,000 psi to produce the cell homogenate. The homogenate was cooled to less than or equal to 10°C prior to each pass through the homogenizer. The homogenate was centrifuged and the solids fraction containing the c105 was retained. The solids were diluted and centrifuged again to give washed inclusion bodies.

The washed inclusion bodies were then dissolved by addition of 8 M urea and 150 mM cysteine in 50 mM TRIS, pH 9.5 This mixture was allowed to stir for two hours at room temperature prior to refolding. Under these conditions, the c105 mutein was denatured and reduced.

The reduced denatured c105 mutein was refolded by dilution with 1.1 M urea, 50 mM Tris to give a final refold solution comprised of 200ug/mL c105 mutein, 1.5 M urea, 7.5 mM cysteine, 50 mM Tris, pH 9.7. The refold mixture was held at 6-10°C for two days. Refold efficiency was monitored by reverse phase HPLC and cation exchange HPLC.

The refold mixture was then brought to pH 5.0 by addition of acetic acid and HCl. The refold mixture was loaded onto a cation exchange column (S-Sepharose big bead resin) previously equilibrated in 25 mM sodium acetate, 65 mM NaCl, pH 5 at 4°C. After loading, the column was washed with the same equilibration buffer. The column was eluted with a gradient from 65 to 350 mM NaCl in 25 mM sodium acetate, pH 5. The c105 mutein eluted at about 200 mM NaCl and was collected in one pool.

The collected pool containing the c105 mutein was diluted with 1.5 volumes of 5 M NaCl, 40 mM sodium phosphate, adjusted to pH 6, and loaded onto a hydrophobic interaction column (Toyo Butyl 650 M column), previously equilibrated in 3 M NaCl, 20 mM sodium

phosphate, pH 6. At the end of the load, the column was washed with equilibration buffer. The c105 mutein was eluted using a linear eight column volume decreasing salt gradient running from 3 M to 1 M NaCl, in 20 mM sodium phosphate, at pH 6. The c105 mutein was collected in one pool. The pool was then concentrated to approximately 3 g/L c105 mutein and then diafilted against 20 mM sodium phosphate, pH 6.0 until the final conductivity was less than 4 mmho (approximately six volumes).

The diafiltered pool was loaded onto a SP-Sepharose high performance column equilibrated in 20 mM sodium phosphate, pH 6.0. After loading, the column was washed with additional equilibration buffer and eluted with a combination pH/salt gradient from 20 mM sodium phosphate, 50 mM NaCl, pH 6.0 to 20 mM sodium phosphate, 50 mM NaCl, pH 6.5. The c105 mutein eluted in the later half of the gradient at about 35 mM NaCl. The c105 mutein can be stored frozen at this point.

The c105 mutein was reacted with the PEGylation reagent at molar ratios of PEG reagent to protein of 1:1, 2:1, 4:1, 1:2 and 0:1 (control). The reaction was carried out in 20 mM phosphate/ 20mM acetate buffer at pH 7.5 for 15 hours at 22°C. Reactions were also carried out in 50mM phosphate buffer, pH 7.5 or 8.5.

The percent conversion to the dumbbell molecule was determined by cation exchange HPLC over a MA7S column. The percent conversion ranged from approximately 40-60%. Conversion to the dumbbell molecule was optimized by adding a solution of approximately 50mg/mL of PEG reagent to the protein at a molar ratio of 0.50-0.65 PEG reagent to 1.0 of TNFbp mutein at pH 7.5 for 15 hours at 22°C. As the ratio of PEG to protein is increased, production of the monoadduct was favored. Monoadduct formation was optimized by a 5:1 ratio of PEG reagent to protein.

Conjugates were purified by chromatography over an S-Sepharose HP column. The reaction mixture was adjusted to pH 3.0-4.2 and loaded onto the column previously adjusted to the same pH. The column was washed with an equilibration buffer and the dumbbell was eluted using a liner sodium chloride gradient and a flow rate of 1.2-1.5 cm/min. The following species eluted from the column in the following order: 1) monosubstituted, 2) dumbbell, 3) unPEGylated TNFbp mutein, and 4) aggregated mutein.

EXAMPLE 11. Bioactivity of TNFbp c105 mutein dumbbell.

c105 dumbbells, whether formed from PEG-*bis* maleimide as described in PCT Application Publication No. WO 92/16221 or as described herein, were shown to be 50 to 100 fold more active than the unPEGylated 30kDa TNF inhibitor by comparison in the L929 cytotoxicity assay set forth in WO 92/16221, incorporated herein by reference.

EXAMPLE 12: Preparation of glyceryl-PEG-tris-vinyl sulfone

Glyceryl-PEG-α,β,γ-triol (10,000 kDa and 20,000 kDa) was converted to the vinyl sulfone derivative using the general method described above. Glyceryl-PEG-α,β,γ-triol was purchased from Union Carbide, Terrytown, New York. Glyceryl-PEG-α,β,γ-triol can be synthesized by ethylene oxide polymerization off of glycerol in base.

EXAMPLE 13: Synthesis of TNFbp c105 trumbbell using glyceryl-PEG-tris-vinyl sulfone

Three TNFbp c105 muteins were conjugated to PEG-tris-vinyl sulfone to yield a "trumbbell" molecule. Experiments conducted over a wide range of PEG:protein ratios showed that a particularly useful molar ratio for conversion to the trumbbell was 0.25-0.35 PEG to 1 protein. In a typical experiment, the c105 mutein in 20mM phosphate, 20mM acetate buffer, pH 7.5 was exposed to a 0.03 molar equivalent of glyceryl-PEG-10,000-α, β, γ-triol at 25°C for 18 hours. Analysis of the latter reaction mixture by cation exchange HPLC (Bio Rad MA7S column eluting a sodium chloride gradient) indicated conversion to the trumbbell in 49% yield and bi-substitution in a 34.9% yield.

EXAMPLE 14: Synthesis of IL-1ra trumbell using glyceryl-PEG-tris-vinyl sulfone.

A solution of PEG-10,000-α,β,γ-tris-vinyl sulfone was reacted with 20 mg/mL wild-type IL-1ra in 0.1 M phosphate buffer at the following PEG/protein molar ratios: 0.10:1; 0.25:1; 0.35:1; 0.45:1; 0.55:1; 0.65:1. The reactions were incubated at 25°C for 72 hours. SDS PAGE analysis showed conversion to mono, di, and triadducted products. Optimal conversion to the triadduct was observed at a PEG/protein ratio of 0.10:1. The reaction mixture was applied to an S Sepharose high performance column and eluted with a sodium chloride gradient.

EXAMPLE 15. Synthesis of c105 TNFbp-PEG-IL-1ra heterodumbbell

A solution of wild-type IL-1ra in 0.1M phosphate buffer, pH 8.5 was reacted with 8 mg/mL PEG-20,000-*bis*-vinyl sulfone-mono-c105TNFbp adduct at the following molar ratios and concentrations of IL-1ra indicated: 55:1 (12.5 mg/mL); 85:1 (18.75 mg/mL); 100:1 (25.0mg/mL) and 150:1 (31.75 mg/mL). After 72 hours, heterodumbbell was formed as determined by SDS PAGE. Optimal conversion was observed at a ratio of 1:100 monoadduct to IL-1ra. The heterodumbbell was purified using an S Sepharose high performance column and eluting with a sodium chloride gradient.

EXAMPLE 16. Stability of PEG-vinyl sulfone polypeptide adducts

The stability of the linkage between the c105 TNFbp mutein and PEG-*bis*-vinyl sulfone was studied. Known amounts of the c105 dumbbell were incubated in PBS, pH7.4, at 37°C for up to one week with aliquots removed at intervals for analysis by SDS PAGE. Essentially no decomposition of the c105 dumbbell was observed. At pH 10 at 37°C for 1 week, only 5-10% degradation of the conjugate was observed.

EXAMPLE 17. TNFbp c105 dumbbell inhibits actively-induced experimental allergic encephalomyelitis ("EAE").

The in vivo activity of the c105 dumbbell made with PEG-*bis*-vinyl sulfone has been demonstrated. EAE is a murine model of an autoimmune inflammatory demyelinating disease of the central nervous system that is often used as a model for human MS. AS described below, the c105 dumbbell inhibited EAE in rats.

Female Lewis rats (150-200g) were purchased from Charles River (Raleigh, NC), and housed for at least 1 week before starting experiments. They received food and water *ad libitum* and were housed in temperature and light controlled (12h/day) rooms. Within each experiment, animals were age-matched.

Active induction of EAE Rats (usually six per group) were anesthetized with 2% isoflurane + O_2 and immunized on day 0 in the footpad of the left hind limb with 0.1 mL of an emulsion containing myelin basic protein ("MBP") at one of the following doses; 0, 1, 3, 10 or 30 μg (fragment 68-84 Bachem Bioscience, PA). The MBP was dissolved in phosphate buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/mL of *Mycobacterium tuberculosis* H37Ra (Difco Lab, MI). Control rats received 0.1 mL of the PBS/CFA emulsion with no MBP in the footpad of the left hindlimb.

Clinical Scoring of EAE Evaluation of clinical disease was performed on a daily basis using a standard 0-5 scoring system. Briefly, the spectrum of rating was 0 normal, 0.5 partial loss of tail tone, 1 complete loss of tail tone, 2 dragging of one hind limb, 3 paralysis of both hind limbs, 4 morbid, and 5 death. Daily weights were recorded for individual rats and weight loss/gain was expressed relative to initial weight.

Effects of immunization with MBP Initial studies assessed the clinical severity of different doses of MBP (0.1-30 μg/ 0.1 mL) in the emulsion described above in the rat. The 0.1 and 0.3 μg MBP doses produced no apparent clinical signs. The 30 ug dose of MBP produced the most severe clinical signs, compared to the 1 ug dose. This effect was highly significant ($p < 0.001$, Mann-Whitney U-test). In general increasing the dose (1-30μg) of MBP produced clinical signs earlier, for example 1ug MBP had a mean $\pm$ S.E.M. onset of 14.88 ± 0.42 (n=9) compared to 12.35 ± 0.16 (n=34; $p<0.01$) days for the 30ug MBP dose. In addition, a dose dependent effect of MBP (1-30ug) on weight loss was observed. Animals spontaneously recovered from the clinical signs within 5-7 days of onset. Administration of CFA alone produced no clinical signs, however, there was an initial transient weight loss compared to non-treated controls.

In all of these studies no significant differences at any of the MBP doses were observed between the no drug (MBP immunized only) and vehicle dosed groups (MBP immunized and dosed with PBS). Thus, vehicle had no effect on the severity of the disease (see Tables 3 and 4). The no drug and vehicle dosed groups are described below.

Treatment of EAE Various doses of TNF inhibitor dumbbell (0.1 - 3 mg/kg) or vehicle (PBS) at various time courses were administered by subcutaneous injection. Treatment periods began either immediately after or nine days after immunization with MBP and continuing until 21 days post immunization. In each experiment, the control rats receiving PBS received the same number of injections as the treatment groups to diminish any secondary effects due to stress. A group of rats receiving no injections whatsoever after EAE induction, the no drug control, was also observed.

Effects of treatment **Every day dosing** The effects on EAE of everyday dosing with the TNF inhibitor dumbbell, starting on the day of immunization for a total of 21 days, was evaluated. Dumbbell concentrations of 0.1, 0.3, 1 or 3mg/kg had no significant effects on reducing severity of the clinical signs in the 1ug and 30ug MBP groups. However, significant amelioration of the clinical disease was observed at the 3ug MBP dose for all dumbbell doses used.

Every other day dosing The effects of 0.1, 0.3, 1 and 3 mg/kg doses given every other day starting on day nine post immunization were also tested. As shown in Tables 3 and 4, a significant inhibition of clinical signs occurred at doses of 0.3 ($p<0.008$), 1.0 ($p<0.001$) and 3.0mg/kg ($p<0.002$, Mann Whitney test, $n=6$) compared to vehicle controls using the highest MBP dose (30ug/0.1mL). No significant differences between the vehicle and the no treatment control groups were observed. The lowest dose of the TNF inhibitor dumbbell had no significant effect on clinical signs.

Dumbbell doses of 1.0 ($p<0.1$) and 3mg/kg ($p<0.05$, Mann Whitney test) significantly attenuated the clinical signs produced by 10ug MBP. Although 0.3 and 0.1mg/kg dumbbell attenuated the clinical signs the reduction was not significant. Dumbbell doses of 0.1-3mg/kg did not significantly inhibit the clinical signs induced by lower doses of MBP (1 or 3ug).

Weight loss is an important marker of EAE onset. Rats immunized with 3, 10, and 30 ug MBP that received the c105 dumbbell (1 or 3mg/kg) lost less weight compared to the vehicle groups.

TABLE 3. PREVENTION OF ACTIVELY-INDUCED EAE WITH TNF INHIBITOR DUMBBELL

TABLES 3A - 3F
EFFECTS OF DUMBBELL ON DAILY MEAN CLINICAL SCORE - 30μg MBP

TABLE 3A

Treatment	no drug						
Mean Clinical Score	0.25 ± 0.18	1.00 ± 0.50	1.92 ± 0.52	2.67 ± 0.44	1.83 ± 0.53	0.83 ± 0.44	0.166 ± 0.10
Days	11	12	13	14	15	16	17

TABLE 3B

Treatment	vehicle						
Mean Clinical Score	0.17 ± 0.17	0.75 ± 0.31	1.83 ± 0.40	2.50 ± 0.34	2.08 ± 0.45	1.00 ± 0.41	0.25 ± 0.11
Days	11	12	13	14	15	16	17

TABLE 3C

Treatment	0.1 mg/kg dumbbell						
Mean Clinical Score	0.08 ± 0.08	0.92 ± 0.35	1.33 ± 0.21	2.67 ± 0.21	2.17 ± 0.30	1.17 ± 0.28	0.25 ± 0.11
Days	11	12	13	14	15	16	17

TABLE 3D

Treatment	0.3 mg/kg dumbbell					
Mean Clinical Score	0.25 ± 0.17	0.92 ± 0.27	1.50 ± 0.42	1.17 ± 0.40	0.58 ± 0.15	0.375 ± 0.14
Days	12	13	14	15	16	17

TABLE 3E

Treatment	1 mg/kg dumbbell					
Mean Clinical Score	0.17 ± 0.17	0.58 ± 0.20	0.67 ± 0.17	0.42 ± 0.20	0.33 ± 0.17	0.083 ± 0.083
Days	12	13	14	15	16	17

TABLE 3F

Treatment	3 mg/kg dumbbell					
Mean Clinical Score	0.25 ± 0.17	0.42 ± 0.20	0.83 ± 0.25	0.42 ± 0.32	0.08 ± 0.08	0.08 ± 0.08
Days	12	13	14	15	16	17

table legend: Daily mean severity score in rats immunized with 30ug MBP and treated with TNF inhibitor dumbbell every other day starting 9 days post MBP-immunization. Vehicle group received PBS and the no drug group received no injections post EAE induction.

TABLE 4. INHIBITORY EFFECTS OF TNF INHIBITOR DUMBBELL EXPRESSED AS AREA UNDER CURVE

Treatment	no drug	vehicle	0.1mg/kg	0.3mg/kg	1mg/kg	3mg/kg
Clinical Severity (Area)	8.07 ± 1.40	7.83 ± 0.88	7.88 ± 0.83	4.3 ± 1.02	1.63 ± 0.60	1.53 ± 1.01

table legend: Inhibitory effects of c105 dumbbell on clinical severity expressed as area under curve (units arbitrary). Mean ±S.E.M. (n=6) were determined for each group and compared statistically against the vehicle group (Mann-Whitney test). No significant differences between the vehicle and no drug control group were observed. c105 dumbbell at 0.3, 1.0 and 3.0 mg/kg (given as described above) significantly (**p <0.008, 0.001, and 0.002 respectively) reduced clinical signs.

As shown in Table 5, every other day dosing also reduced the duration of the disease as measured by the number of days during which any clinical signs were observed and the mean calculated for a given group of rats.

TABLE 5. DURATION OF THE DISEASE WITH EVERY OTHER DAY DOSING

MBPμg	TNF inhibitor dumbbell mg/kg				
	0	0.1	0.3	1	3
30	5.33 ± 0.21	5.50 ± 0.34	4.50 ± 0.92	2.83 ± 0.79*	2.16 ± 0.60**
10	4.33 ± 0.80	3.66 ± 0.80	4.00 ± 0.51	3.33 ± 0.49	1.83 ± 0.70*
3	2.50 ± 1.02	1.83 ± 0.83	2.00 ± 0.81	3.16 ± 0.74	0.83 ± 0.54
1	1.83 ± 0.79	0.66 ± 0.66	1.66 ± 0.61	1.33 ± 0.49	0.66 ± 0.42

* $p < 0.05$ ** $p < 0.01$

Single dosing A single dose of either 0.3 or 3mg/kg dumbbell administered on day nine post immunization had little or no effect on attenuating MBP (1-30μg) induced clinical signs when compared to vehicle controls.

Every third day administration of TNF inhibitor dumbbell Dumbbell at 0.1-3mg/kg or vehicle was administered on days 9, 12, 15 and 18 post MBP-immunization. As shown in Table 6, a significant attenuation of MBP (30μg) induced clinical signs was observed at c105 dumbbell doses of 0.3 ($p<0.05$), 1.0 ($p<0.01$) and 3mg/kg ($p<0.001$ Mann-Whitney t-test). The 0.1mg/kg dose of c105 dumbbell was without effect when compared to the vehicle control.

The MBP (10μg) induced clinical signs were reduced by 0.3, 1.0 and 3.0mg/kg c105 dumbbell doses. However, significant ($p<0.05$ and 0.03 respectively) effects were only observed at the higher c105 dumbbell doses. Although c105 dumbbell (0.3-3mg/kg) reduced the clinical signs produced by 3ug of MBP by approximately 20-60%, the effects observed were not significantly different from the vehicle control group.

The duration of the disease was generally reduced by c105 dumbbell. For example, c105 dumbbell at 1 and 3mg/kg significantly reduced the duration MBP (30μg) mediated signs by 37.3% and 68.7% respectively (see Table 10). A similar trend was also observed using the intermediate MBP (10μg) dose but not the lowest MBP dose (Table 7).

Disease onset in the 10 and 30 μg MBP groups were significantly ($p < 0.047$; $p < 0.013$ respectively; Mann Whitney U-test) delayed in those animals that were treated with 3mg/kg c105 dumbbell.

The weight loss associated with EAE was partially inhibited by c105 dumbbell especially at the 1 and 3mg/kg doses. The reduction in weight loss was dose dependent. This effect of c105 dumbbell was similar no matter what dose of MBP was used.

TABLE 6. MEAN CLINICAL SEVERITY EXPRESSED AS AREA FOR EVERY THIRD DAY DOSING

Treatment	Vehicle	0.1mg/kg	03.mg/kg	1.0mg/kg	3.0mg/kg
Mean Clinical Severity (Area)	9.21 ±0.64	8.25 ±0.92	6.23 ±1.37	3.66 ±0.61	0.33 ±0.17

TABLE 7. DURATION OF THE DISEASE WITH EVERY THIRD DAY DOSING

MBPμg	TNF inhibitor dumbbell mg/kg				
	0	0.1	0.3	1	3
30	5.83 ± 0.44	4.83 ± 0.30	4.16 ± 0.70	3.66 ± 0.61*	1.83 ± 0.70**
10	4.66 ± 0.42	5.16 ± 0.40	4.00 ± 0.77	3.00 ± 0.96	2.50 ± 0.67*
3	4.00 ± 0.67	3.50 ± 0.62	3.00 ± 1.35	3.00 ± 1.35	3.33 ± 0.66

* $p < 0.05$ ** $p < 0.01$

EXAMPLE 18. Central Nervous System (CNS) Pathology

The effects of treatment with c105 dumbbell synthesized using PEG-*bis*-vinyl sulfone were determined on CNS pathology induced by immunization with MBP (0, 10 or 30μg). MBP-immunization (EAE induction) was performed as described above. c105 dumbbell at 0.3, 3mg/kg or vehicle was administered every other day beginning on day nine post MBP. Animals were killed (via CO_2) on days 9, 14 or 20 post-MBP injection. The brain and spinal cord from each rat were removed and placed in 10% neutral buffered formalin. Following fixation for at least 72 hours, cross sections of the brain were made at the level of the optic chiasm caudal to the attachment of the pituitary and the transverse fibers of the pons. The spinal cord was trimmed by making 4-6 cross sections through the cervical, thoracic and lumber portions. The sacral segment with attached caudal nerves was embedded longitudinally. Tissues were processed for paraffin embedding and stained with hematoxylin and eosin.

Histologic evaluations were done without knowledge of the treatment groups. Each slide was assigned a numerical score ranging from 1-4 to indicate the intensity of inflammation and demyelination. Scoring criteria were as follows; 1=minimal 1-2 vessels

have small perivascular cuffs of inflammatory cells, 2=mild 3 or more vessels have small perivascular cuffs of inflammatory cells with little if any extension of inflammation into parenchyma, 3=moderate 3 or more vessels have prominent perivascular cuffs of inflammatory cells with moderate extension of the inflammation into the surrounding parenchyma, and 4=marked the majority of vessels have prominent perivascular cuffs of inflammatory cells with extensive involvement of the neuropil in the inflammatory process.

Total inflammation scores were determined for each of animals for each CNS region. Mean ± SEM (standard error of the mean) score values were computed for each portion of the CNS for each time point and compared against the vehicle treated animals.

The mean inflammatory score were determined for each CNS region for each group of animals and compared statistically against the vehicle control group (students-t-test). These scores are set forth in Tables 8 and 9.

There were no significant histologic alterations in the CNS of animals killed at day 9 post-MBP injection. Lesions at day 14 consisted of minimal to marked mixed (mononuclear + some neutrophils) generally perivascular inflammatory cell infiltration. In the brain, the inflammation tended to be located in the meninges, periventricular areas and cerebellar white tracts, with the brain stem and cerebellar white tracts being most severely affected. In these locations, the inflammation often extended from perivascular areas into the surrounding parenchyma and there was evidence of demyelination. Within the spinal cord, the lumbar and sacral portions were most severely affected. Both gray and white matter were affected, again with the predominant lesion being perivascular. Inflammation persisted into day 20, however, neutrophils were rarely seen at this time point. Variability in intensity of inflammation occurred within animals in each group and almost all group.

Tables 8 and 9 demonstrate the presence of c105 dumbbell reduced the degree of inflammation in the various regions of the CNS studied. The most dramatic and significant reductions in inflammation were observed in the spinal cord, particularly the lumbar and sacral regions. c105 dumbbell had a lesser effect on the higher regions of the CNS, cerebrum and cerebellum.

TABLE 8. INFLAMMATORY SCORES OF ANIMALS IMMUNIZED WITH 30UG MBP AND TREATED WITH TNF INHIBITOR DUMBBELL

Brain Region	3mg/kg	0.3mg/kg	Vehicle
Cerebrum	1.00 ± 0.378	0.714 ± 0.360	0.714 ± 0.474
Cerebellum	2.57 ± 0.429	2.714 ± 0.360	3.280 ± 0.286
Cervical cord	1.71 ± 0.360	1.428 ± 0.298*	2.420 ± 0.202
Thoracic cord	1.71 ± 0.421	1.000 ± 0.218*	2.280 ± 0.421
Lumbar cord	1.85 ± 0.404	1.42 ± 0.369	2.42 ± 0.298
Sacral cord	1.28 ± 0.360	1.42 ± 0.298	2.714 ± 0.522

* $p < 0.05$ (Students t-test) Histology (30ug MBP dose)

TABLE 9. INFLAMMATORY SCORES OF ANIMALS IMMUNIZED WITH 10UG MBP AND TREATED WITH TNF INHIBITOR DUMBBELL

Brain Region	3mg/kg	0.3mg/kg	Vehicle
Cerebrum	0.28 ± 0.18	0.42 ± 0.20	0.42 ± 0.29
Cerebellum	1.42 ± 0.29	2.28 ± 0.42	2.28 ± 0.35
Cervical cord	0.85 ± 0.34	1.42 ± 0.42	1.42 ± 0.20
Thoracic cord	0.85 ± 0.14	1.57 ± 0.36	1.0 ± 0.30
Lumbar cord	0.71 ± 0.28**	1.57 ± 0.48	2.28 ± 0.28
Sacral cord	0.57 ± 0.20**	1.57 ± 0.48	2.28 ± 0.42

** $p < 0.01$ Histology (10ug MBP dose)

EXAMPLE 19. c105 TNFbp dumbbell protects against endotoxin lethality

The c105 dumbbell synthesized using PEG-*bis*-vinyl sulfone protected Balb/c mice against a lethal dose of endotoxin. Mice were injected intraperitoneally with 30 mg/kg endotoxin and intravenously with a single administration of either 0.1 mL PBS or 1 mg/kg dumbbell in 0.1 mL PBS at either 1 hour or two hours after the administration of endotoxin. The intravenous administration of 1 mg/kg dumbbell 1 hour after injection of endotoxin caused almost complete protection against lethality. Dumbbell administration at the two hour time point gave no protection against the lethal endotoxin injury.

The c105 dumbbell also protected Lewis rats against a lethal dose of endotoxin. Rats were injected intravenously with 12.5 mg/kg endotoxin. Rats were injected simultaneously with endotoxin and either saline or the c105 dumbbell at doses of either 0.1, 0.5, 3.0 or 4.5 mg/kg. Comparable protection against lethal injury was achieved at all dumbbell doses.

A single dose treatment of 1.5 mg/kg c105 dumbbell given simultaneously with a 10 mg/kg dose of endotoxin protected rats against hepatic and metabolic disturbances. Hepatic

and metabolic parameters were assessed at 24 hours after the administration of endotoxin as shown in Table 10.

TABLE 10: EFFECTS OF TREATMENT WITH c105 DUMBBELL (1.5 MG/KG) ON ENDOTOXIN-INDUCED ABNORMALITIES IN BIOCHEMICAL PARAMETERS

Parameter	Control + Vehicle	Endotoxin + Vehicle	Endotoxin + c105 dumbbell
Glucose (mg/dL)	143 ± 2	52 ± 8	81 ± 5*
SGPT[1] (mu/mL)	47 ± 6	679 ± 118	141 ± 25*
Blood Urea Nitrogen (mg/dL)	19 ± 1	88 ± 2	39 ± 3*
Corticosterone (ng/mL)	164 ± 62	750 ± 49	489 ± 43*

[1] Serum Glutamic Pyruvic Transaminase
Values are means ± standard error for 4 to 8 rats per group.
*Significantly different from the endotoxin-treated group at $p < 0.05$ (paired t test)

It is to be understood that the application of the teachings of the present invention to a specific expression system or PEGylation reagent will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Thus, it will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the process and products of the present invention. It is intended that the present invention covers these modifications and variations.

What is claimed is:

1. A biologically-active conjugate comprising a biologically-active molecule having a reactive thiol moiety and a non-peptidic polymer having an active sulfone moiety forming a linkage with said thiol moiety.

2. The biologically-active conjugate of claim 1, wherein said active sulfone moiety is vinyl sulfone.

3. The biologically-active conjugate of claim 1, wherein said active sulfone moiety is chloroethyl sulfone.

4. The biologically-active conjugate of claim 1, wherein said biologically-active molecule is a tumor necrosis factor (TNF) inhibitor.

5. The biologically-active conjugate of claim 4, wherein said TNF inhibitor is selected from the group consisting of a 30kDa TNF inhibitor, a 40kDa TNF inhibitor, a Δ51 TNF inhibitor, and a Δ53 TNF inhibitor.

6. The biologically-active conjugate of claim 5, wherein said TNF inhibitor is the 30kDa TNF inhibitor.

7. The biologically-active conjugate of claim 5, wherein said TNF inhibitor is the 40kDa TNF inhibitor.

8. The biologically-active conjugate of claim 5, wherein said TNF inhibitor is the Δ51 TNF inhibitor.

9. The biologically-active conjugate of claim 5, wherein said TNF inhibitor is the Δ53 TNF inhibitor.

10. The biologically-active conjugate of claim 1, wherein said biologically-active molecule is an interleukin-1 (IL-1) inhibitor.

11. The biologically-active conjugate of claim 10, wherein said IL-1 inhibitor is interleukin-1 receptor antagonist (IL-1ra).

12. A method of preparing a biologically-active conjugate, comprising the steps of:

(a) reacting a biologically-active molecule having a reactive thiol moiety with a non-peptidic polymer having an active sulfone moiety to form said conjugate; and

(b) optionally, isolating said conjugate.

13. The method of claim 12, further comprising, before step (a), the steps:

selecting a desired biologically-active molecule; and

adding a reactive thiol moiety to the selected molecule to form a biologically-active molecule having a reactive thiol moiety.

14. The method of claim 12, further comprising, before step (a), the steps of:

selecting a biologically-active molecule;

adding a reactive thiol moiety to the selected molecule to form a synthetic molecule; and

refolding the synthetic molecule to form a biologically-active molecule having a reactive thiol moiety; and

optionally, isolating the biologically-active molecule having a reactive thiol moiety.

15. A substantially purified compound of the formula R_1-X-R_2, wherein:

X comprises a non-peptidic polymer having a first reactive group and a second reactive group, wherein said first reactive group is a Michael acceptor;

R_1 comprises a biologically-active molecule having a reactive thiol moiety, said biologically-active molecule is covalently bonded to said non-peptidic polymer by reaction of said thiol moiety with said Michael acceptor, and said biologically-active molecule retains its biological activity after said reaction; and

R_2 comprises a biologically-active molecule or a nonbiologically-active group bonded to said non-peptidic polymer by reaction with said second reactive group.

16. The substantially purified compound of claim 15, wherein said Michael acceptor is vinyl sulfone.

17. The substantially purified compound of claim 15, wherein said Michael acceptor is maleimide.

18. The substantially purified compound of claim 15, wherein said non-peptidic polymer has two Michael acceptors.

19. The substantially purified compound of claim 18, wherein said Michael acceptors are maleimide.

20. The substantially purified compound of claim 18, wherein said Michael acceptors are vinyl sulfone.

21. The substantially purified compound of claim 18, wherein one of said Michael acceptors is vinyl sulfone and the other is maleimide.

22. The substantially purified compound of claim 15, wherein said biologically-active molecule is selected from the group consisting of an IL-1 inhibitor, a tumor necrosis factor binding protein (TNFbp), CR1, PDGF receptor, IL-2, and exon 6 peptide of PDGF.

23. The substantially purified compound of claim 22, wherein said biologically-active molecule is a tumor necrosis factor binding protein (TNFbp).

24. The substantially purified compound of claim 23, wherein said TNFbp is the 30kDa TNF inhibitor.

25. The substantially purified compound of claim 23, wherein said TNFbp is the 40kDa TNF inhibitor.

26. The substantially purified compound of claim 23, wherein said TNFbp is the Δ51 TNF inhibitor.

27. The substantially purified compound of claim 23, wherein said TNFbp is the Δ53 TNF inhibitor.

28. The substantially purified compound of claim 22, wherein said biologically-active molecule is an IL-1 inhibitor.

29. The substantially purified compound of claim 28, wherein said IL-1 inhibitor is IL-1ra.

30. The substantially purified compound of claim 15, wherein R_1 comprises a biologically-active polypeptide.

31. The substantially purified compound of claim 15, wherein R_1 and R_2 comprise biologically-active polypeptides.

32. The substantially purified compound of claim 15, wherein R_1 and R_2 are identical.

33. The substantially purified compound of claim 15, wherein R_1 and R_2 are different.

34. A water-soluble polymer having a reactive NHS-ester and a reactive Michael acceptor.

35. The water-soluble polymer of claim 34, wherein said Michael acceptor is vinyl sulfone.

36. The water-soluble polymer of claim 34, wherein said Michael acceptor is maleimide.

37. The water-soluble polymer of claim 34, wherein said polymer is selected from the group consisting of polyalkylene oxides, polyoxyethylated polyols, and polyolefinic alcohols.

38. A method for the preparation of the substantially purified compound of claim 15, comprising:

(a) reacting X with R_1 and R_2 to form R_1-X-R_2; and

(b) purifying R_1-X-R_2.

39. The method of claim 38, further comprising adding a reactive thiol moiety to a biologically-active molecule to form R_1 prior to step (a).

40. The method of claim 38, further comprising, prior to step (a), the steps:

selecting a biologically-active molecule;

adding a reactive thiol moiety to the selected molecule to form a synthetic molecule;

refolding the synthetic molecule to form R_1; and

optionally, isolating R_1.

41. The method of claim 38, wherein step (a) further comprises the steps:

protecting a reactive group of X to form a protected group on X;

reacting X having a protected group with R_1 to form R_1-X;

deprotecting the protected group on X;

reacting R_1-X with R_2 to form R_1-X-R_2.

42. The method of claim 38, wherein step (a) further comprises the steps:

reacting an excess of X with R_1 to form R_1-X; and

reacting R_1-X with R_2 to form R_1-X-R_2.

43. A pharmaceutical composition comprising the compound of claim 1 in a pharmaceutically-acceptable carrier.

44. A pharmaceutical composition comprising the compound of claim 15 in a pharmaceutically-acceptable carrier.

Abstract of the Disclosure

Biologically active conjugates are disclosed which are formed by reaction of a thiol moiety of a biologically active molecule with a non-peptidic polymer having an active sulfone moiety. Also disclosed are compounds having the formula R_1-X-R_2 wherein at least one of R_1 and R_2 is a biologically active molecule having a reactive thiol moiety which forms a covalent bond with X, a Michael acceptor-activated non-peptidic polymer. Further disclosed are methods of making the conjugates and compounds of the present invention as well as pharmaceutical compositions containing them. In addition, activated polymers suitable for attachment to a variety of molecules and surfaces are disclosed.